Adaptation or physiological constraint: Temperature-mediated plasticity in reproduction

Marc Johan Steigenga

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vorgelegt von

Marc Johan Steigenga

Bayreuth

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Prof. Dr. Klaus H. Hoffmann (Erstgutachter)
Prof. Dr Klaus Fischer (Zweitgutachter)

Prof. Dr. Gerhard Rambold (Vorsitzender)
Prof. Dr. Ingolf Steffan-Deventer
PD Dr. Matthias W. Lorenz

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1

Introduction

1.1. Introduction

Investigating interactions between organisms and their environment has been at the forefront of biological research ever since Darwin's realisation (1859) that the latter plaid an important role in shaping the former (Barnes & Partridge 2003). It has been recognised that all existing organisms are the results of a long evolutionary history in which natural selection is believed to play the main part in shaping the organisms phenotypes (Barnes & Partridge 2003). Life history theory (e.g. the age-specific schedule of fecundity and mortality, Barnes & Partridge 2003) relates an individual's phenotype to its fitness and lies therefore at the heart of biology, and is further needed to understand the action of natural selection. It also helps us to understand how the other central element, genetic variation, impacts on phenotypes. Life history traits figure directly in reproduction and survival and include - amongst many others - growth trajectories, age and size at maturity, number and size of offspring, age and size specific reproductive investment and mortality schedules (Stearns 1992). For natural selection to act on these traits, two prerequisites are necessary. First, heritable variability for the trait in question determines whether there will be a response to selection and second, individuals (phenotypes) must vary in fitness (Scheiner & Lyman 1991; Stearns 1992; Falconer & Mackay 1996; Ernande *et al.* 2004).

Two sources of phenotypic variation in life-history traits have long been recognized, namely genetic differentiation and effects of different environments on the expression of the phenotype (Schmalhausen 1949; Endler 1986). The latter source of variation, called phenotypic plasticity refers to cases when a single genotype can produce alternative phenotypes. Such plastic changes may merely represent a biochemical or physiological interaction of the organism with its environment, or it may be an adaptation to spatially heterogeneous or temporarily varying environments (adaptive phenotypic plasticity) (Levins 1963; Bradshaw 1965: Nylin & Gotthard 1998).

Phenotypic plasticity has two important roles in evolution. First, by modifying the relationship among traits and trait fitness, it changes the selection pressures on traits across environments. Second, by modulating the expression of genetic variation and of genetic covariation it shields the genotype from the effects of selection (Stearns 1992; Falconer & Mackay 1996). However, phenotypic plasticity is also a property of

the genotype and there is genetic variation for plastic responses (Pigliucci 2005). While selective forces were presumed to be omnipotent in shaping phenotypes, the evolution of traits or their combinations may only be partially realised due to counteracting properties or mechanisms limiting or channelling responses to selection (Stearns 1992; Roff 2002; Barnes & Partridge 2003). The general consensus is that life histories must involve compromises between what selection can achieve (adaptation) and what selection is prevented from achieving (constraints; reviewed in Barnes & Partridge 2003). These constraints are often due to lack of sufficient genetic variation in plasticity and costs associated with phenotypic plasticity (e.g. maintenance, production, pleiotropy, epistasis, De Witt *et al*. 1998).

However, in recent years it has become evident that the individual phenotype is often also affected by the environmental experience of other individuals (Mousseau & Dingle 1991; Mousseau & Fox 1998). In general inter-individual interactions occur most frequently between parents (primarily mothers) and their offspring (Mousseau & Fox 1998; Weigensberg *et al*. 1998; Wolf *et al*. 1998; Amarillo-Suárez & Fox 2006). In many organisms, a female's environment may provide a reliable indicator of the environmental conditions their offspring will encounter. In such cases, maternal effects may evolve as mechanisms for 'trans-generational' phenotypic plasticity (Mousseau & Dingle 1991; Fox & Mousseau 1998; Rossiter 1996) whereby in response to a predictive environmental cue a mother can tune her offspring's phenotype for that environment (i.e. adaptive phenotypic plasticity; Fox *et al*. 1997; Wolf *et al*. 1998; Gilchrist & Huey 2001).

Such non-genetic influences of parental phenotype or environment on progeny phenotype are of evolutionary importance not only because they influence short-term responses to selection (Kirkpatrick & Lande 1989), but also because they are potentially adaptive (Mousseau & Dingle 1991; Rossiter 1996; Fox *et al*. 1997). Environmental experience can be transmitted to offspring via cytoplasmic egg factors, e.g. yolk amount, egg composition, hormones or mRNA (Fox & Mousseau 1998; Mousseau & Fox 1998; Sakwinska 2004).

1.2. Why study reproduction?

Phenotypic correlations between egg size and number among species, among populations within species, and among individuals within populations generally indicate a trade-off between egg size and number in arthropods (Fox & Czesak 2000). Larger offspring were found to have a higher juvenile survivorship, faster maturation, increased survival under stressful conditions and improved competitive abilities as compared to small offspring (Azevedo *et al*. 1997; Fox & Czesak 2000; Czesak & Fox 2003; Fischer *et al*. 2003a). At the same time the fitness of the mother increases with increasing progeny numbers, thus favouring more but smaller offspring, within the limits posed by offspring viability (Azevedo *et al*. 1997; Fox & Czesak 2000). This conflict of interest between parents and progeny leads to an optimal egg size, balancing maternal and offspring selection (Smith & Fretwell 1974). Therefore, egg size is an especially interesting life history trait, as it is simultaneously a maternal and a progeny character (Fox & Czesak 2000).

One of the most striking and best-described phenomena with regard to variation in insect egg size is temperature-mediated plasticity. Eggs of ectothermic animals were commonly found to be larger in colder regions and at colder times, and under laboratory conditions females usually lay larger eggs at lower temperatures (e.g. Azevedo *et al.* 1996; Crill *et al*. 1996; Yampolski & Scheiner 1996; Ernsting & Isaaks 1997; Blanckenhorn 2000; Atkinson *et al.* 2001; Fischer *et al*. 2003a, 2006a,b).

The capacity to produce a given number of offspring resides primarily in the number of ovarioles/ovaries, ovariole structure, and longevity of the species. Photoperiod and temperature are the most important environmental factors influencing the process of reproduction and the release of various reproductive hormones (Nijhout 1998). In insects, hormones are the main regulators of life-history components like metamorphosis, behaviour, caste determination, diapause, polymorphisms and reproduction (Edwards *et al*. 1995; Gäde *et al*. 1997; Nijhout 1998; Flatt *et al.* 2005). The principle hormones influencing these components are the juvenile hormones and the ecdysteroids. As the biosynthesis and regulation of juvenile hormone depends on environmental conditions such as temperature and photoperiod, they are frequently involved in mediating phenotypic plasticity (e.g. Dingle & Winchell 1997; Zera *et al*. 1998; Emlen & Nijhout 1999). Differences in juvenile hormone titres in turn can affect reproductive output (e.g. Cusson *et al*. 1990; Trumbo & Robinson 2004).

Although resource allocation trade-offs have been successfully used as a conceptional tool for modelling the evolution of life histories, their mechanic and hormonal base have rarely been analysed (Ernsting & Isaaks, 2000; Barnes & Partridge 2003).

1.3. Rationale of this thesis

Using the tropical butterfly *Bicyclus anynana* as a model organism this study focuses on two main themes concerning phenotypic plasticity: **reproductive phenotypic plasticity** and its **mechanistic and hormonal basis**. Both are tightly linked and assumed to cause variation in offspring traits, affecting maternal and offspring fitness (Fox & Czesak 2000; Czesak & Fox 2003; Fischer *et al*. 2003a, 2006).

Currently, the most serious lack in our understanding of phenotypic plasticity is experimental studies on the genetic basis of phenotypic plasticity (Scheiner & Lyman, 1991; Scheiner, 1993) as well as the influence of epigenetic cross-generational effects of temperature (Zamudio *et al*. 1995; Crill *et al*. 1996; Huey & Berrigan 1996; Gilchrist & Huey 2001; Stillwell & Fox 2005).

This first part of the thesis (**chapter 5**) investigates whether there is sufficient standing genetic variation in temperature-related plasticity for short-term evolutionary change to occur (**subchapter 5.1**), and the influence of cross-generational effects on reproductive phenotypic plasticity (**subchapter 5.2**).

While the effects of juvenile hormone on ovarian and egg development are fairly well understood (for Lepidoptera e.g. Pan & Wyatt 1971, 1976; Herman & Bennett 1975; Satyanarayana *et al*. 1991, 1992), data on its effects on reproductive output are scarce (Rankin *et al*. 1997; Trumbo & Robinson 2004), especially in Lepidoptera (e.g. Ramaswamy *et al*. 1997; Webb *et al*. 1999).

The above mentioned almost universal pattern of larger body, egg, or cell size at lower temperatures is usually referred to as the temperature-size rule (Atkinson, 1994; Atkinson *et al*., 2001). However, we do not yet understand the underlying mechanisms shaping phenotypic plasticity in reproduction (Azevedo *et al*. 1996; Crill

et al. 1996; Blanckenhorn 2000; Fox & Czesak 2000; Fischer *et al*. 2003a; Walters & Hassall 2006), with multiple competing hypothesis trying to explain the observed reproductive phenotypic plasticity (Wallin *et al*. 1992; Avelar 1993; Moore & Folt 1993; Huey *et al*. 1995; Van der Have & De Jong 1996; Van Voorhies 1996; Ernsting & Isaaks 2000; Fox & Czesak 2000; Blanckenhorn & Henseler 2005).

The second part (**chapter 6**) of the thesis thus investigates the hormonal basis of the observed phenotypic plasticity (**subchapter 6.1**) as well as the ovarian dynamics in addition to egg size and number and the relevance of the competing hypotheses (**subchapter 6.2**).

1.4. Study organism - the tropical butterfly *Bicyclus anynana*

This study uses *Bicyclus anynana* (Butler 1879), a tropical, fruit-feeding butterfly with a distribution ranging from Southern Africa to Ethiopia (Larsen 1991). The species exhibits striking phenotypic plasticity (two seasonal morphs), which is thought to function as an adaptation to alternative wet-dry seasonal environments and the associated changes in resting background and predation (Brakefield 1997; Lyytinen *et al*. 2004). Reproduction in this species is essentially confined to the warmer wet season when oviposition plants are abundantly available for oviposition. During the wet season two overlapping generations occur, the first arising from eggs laid by the dry season form during rising temperatures, and the second generation from wet season butterflies (Brakefield & Reitsma 1991). Butterflies of the second generation lay eggs towards the end of the wet season when there is a marked decrease in temperature, starting several weeks before the larval food plants dry out (*Oplismenus* and *Setaria grasses*, up to 90% brown). They give rise to progeny of the dry season form, which feed on the increasingly decaying grasses (Brakefield & Reitsma 1991). During this colder and dryer season reproduction ceases and the butterflies do not mate before the rains of the next wet season (Brakefield 1997; Windig 1994). As morphs are gradually replaced during transitions between the seasons, both phenotypes may occur simultaneously (Brakefield & Reitsma 1991). Apart from changes in wing coloration, dry season morphs differ from wet season morphs in multiple ways including increased egg and body weight, larger fat bodies and a reproductive diapause to survive the harsh dry season (Brakefield & Reitsma 1991).

A laboratory stock population of *B. anynana* was established at Bayreuth University, Germany, in 2003 from several hundred eggs derived from a well-established stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from over 80 gravid females caught at a single locality in Malawi. Several hundred adults are reared in each generation, maintaining high levels of heterozygosity at neutral loci (Van't Hof *et al*. 2005). For this study butterflies from the Bayreuth stock population were used.

2

Synopsis

2.1. The evolutionary genetics of egg size plasticity in a butterfly

Currently, the most serious lack in our understanding of phenotypic plasticity is experimental studies on its genetic basis (Scheiner & Lyman 1991; Scheiner 1993). Recent studies showed that *B. anynana* females kept at a lower oviposition temperature laid larger but fewer eggs than those kept at a higher temperature (Fischer *et al*. 2003a,b,c, 2004). The existence of genotype-environment interactions in this plastic response would be indicative of the potential for short term evolutionary change, and thus natural selection should be able to reshape the plastic response to temperature (Roskam & Brakefield 1999).

Here, I set out to test for the existence of genetic variation in the plastic response of egg size to temperature in *Bicyclus anynana* (Butler 1879), employing a half-sib breeding design, to explore genetic variation in temperature reaction norms (e.g. Falconer & Mackay 1996).

In agreement with earlier studies using *B. anynana* as a model organism (Fischer *et al.* 2003a,b,c, 2004), temperature clearly induced a plastic response in egg size as a direct consequence of differences in the oviposition environment. The interactions between the plastic response in egg size between full- or half sib families suggest the existence of genetic variation in the plastic response to temperature and thus the potential for short-term evolutionary change. This genotype-environment interaction is graphically evident in crossing of the temperature reaction norms in Figure 1. While most of the full-sib families follow the expected pattern of an increase in egg size with time, some families behave differently in showing virtually no response. In addition to the genetic variation in reaction norms, cross-environmental correlations, although being positive, were significantly less than unity suggesting that any constraints on evolutionary potential due to genetic correlations are unlikely to prevent evolution to new phenotypic values in different environments (although they may reduce the rate of such evolution) (Beldade *et al*. 2002; Zijlstra *et al*. 2004).

Additive genetic effects on egg size were weak and differed quite substantially from other estimates of egg size heritability in *B. anynana* (e.g. Fischer *et al.* 2004). The heritability of egg size in *B. anynana* is overall low to moderate, however, as

expected for a typical life-history trait (Roff 2002). Overall, additive genetic effects of the male on egg size were weak, as judged by the low estimates for additive genetic variance. Effects of the female, in contrast, were highly significant throughout and maternal effect variance explained 10 to 14 % of the total phenotypic variance in egg size. These results suggest the existence of either non-additive genetic variances or rather strong effects of the female's environment, phenotype or genotype on egg size. In contrast, Czesak and Fox (2003) found that egg size in the seed beetle *Stator limbatus* was only marginally affected by maternal effects variance. My results suggest that either non-additive genetic variance exists or that egg size is rather strongly affected by the female's environment, phenotype or genotype.

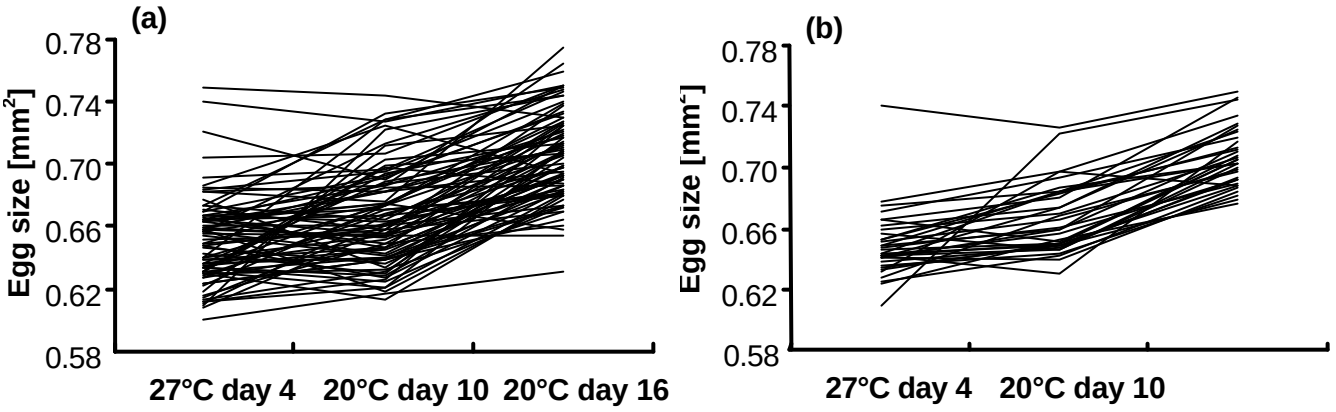

Figure 1. *Reaction norms for egg size in relation to adult temperature in the butterfly Bicyclus anynana. Each line represents the mean values of a full-sib (a) or half-sib family (b), respectively. Measurements took place on days 4, 10 and 16 of the females' adult life span. Females were transferred from 27 to 20°C on day 4.*

The most parsimonious explanation is that the maternal effects are due to female genotype. It is well known from other animals that even within single clones reared in a common environment, maternal identity effects can have a profound influence on offspring size (e.g. in *Daphnia;* Sakwinska 2004), and also the data from parent-offspring regressions suggest that maternal effects are important. Maternal environmental effects may evolve for cross-generational phenotypic plasticity, with mother's passing on their experience to the offspring to increase offspring fitness in predictable environments (Mousseau & Dingle 1991; Rossiter 1996; Fox & Mousseau 1998; Mousseau & Fox 1998). Although cross-generational maternal effects could potentially occur in *B. anynana* as this butterfly lives in a highly predictable

environment with distinct seasons (Brakefield 1997), such a mechanism is unlikely to account for the current results as all butterflies were reared for many generations under identical conditions.

In summary, my results demonstrate that in the butterfly, *B. anynana,* egg size responds in a plastic manner to oviposition temperature, that egg size is heritable and that there seems to be genetic variation in the plastic response to temperature. These findings, together with earlier ones suggesting that temperature-mediated egg size plasticity in this particular species is adaptive (Fischer *et al.* 2003a,c), suggest that the conditions necessary for the evolution of phenotypic plasticity to occur are fulfilled.

2.2. Within- and between-generation effects of temperature on life-history traits

Although the potential importance of parental (mainly maternal) effects is well established, there is still a relative lack of studies looking explicitly at such effects (Wolf *et al*. 1998, Lindholm *et al*. 2006), and fairly little is known about cross-generational effects of temperature (Zamudio *et al*. 1995; Crill *et al*. 1996; Huey & Berrigan 1996; Gilchrist & Huey 2001; Stillwell & Fox 2005).

I have investigated within- and between-generation effects of temperature on development time, pupal mass and egg size in the tropical butterfly *Bicyclus anynana* (Butler 1879) by raising butterflies at high and low parental temperature, and afterwards for one generation at high and low developmental temperature. Additionally, ovipositing females were divided among two adult (i.e. acclimation) temperatures.

In this study I found substantial effects of developmental and acclimation temperature on life-history traits (i.e. within-generation effects; see below), but also of the temperature experienced in the parental generation (i.e. between-generation or carry-over effects). Parental carry-over effects are usually most pronounced early in life and diminish when offspring matures (e.g. Mousseau & Dingle 1991; Crill *et al.* 1996; Wolf *et al*. 1998; McAdam *et al*. 2002; Sakwinska 2004, Lindholm *et al*. 2006).

This notion agrees with my results as larval development time and growth rate were strongly affected by parental temperature in *B. anynana* (cf. Roff & Sokolovska 2004). Additionally, pupal time and mass were affected across generations, which seems to be quite rare in animals (but Crill *et al*. 1996; Sakwinska 2004), though environmentally-induced phenotypic variation across multiple generations has been detected in many studies with plants (Bernardo 1996). Regarding effect directions, animals whose parents had been raised at the lower temperature had longer larval and pupal times, a concomitantly reduced larval growth rate, and an increased pupal mass compared to those whose parents had been raised at the higher temperature (Figure 2). These patterns are in broad agreement with those previously reported for *Drosophila* (Crill *et al*. 1996; Gilchrist & Huey 2001), and closely resemble the effects typically induced by differences in developmental temperature (Atkinson 1994; Partridge & French 1996; Chown & Gaston 1999; Atkinson *et al*. 2001; Fischer *et al*. 2003a).

As expected, development times were much longer and larval growth rates much lower when animals were reared at a lower temperature (see above; Honek & Kocourek 1990; Fischer *et al*. 2003a). Contrary to my expectations based on the temperature-size rule (e.g. Atkinson 1994), however, pupal mass was not significantly affected by rearing temperature and even tended to increase at the higher temperature. This may suggest that, regarding pupal mass as opposed to development times, parental and developmental temperature did not interact in a synergistic, but rather antagonistic manner (see above; see also Crill *et al*. 1996). The significant interactions between parental and rearing temperature for larval time, pupal time and growth rate indicate that rearing temperature effects depend at least to some extent on the environment experienced by the parents. Regarding egg size, females reared at the lower temperature produced larger eggs compared to those being reared at the higher temperature (Avelar 1993; Ernsting & Isaaks 1997; Blanckenhorn 2000; Fischer *et al*. 2003b, 2004, 2006b) (Figure 3).

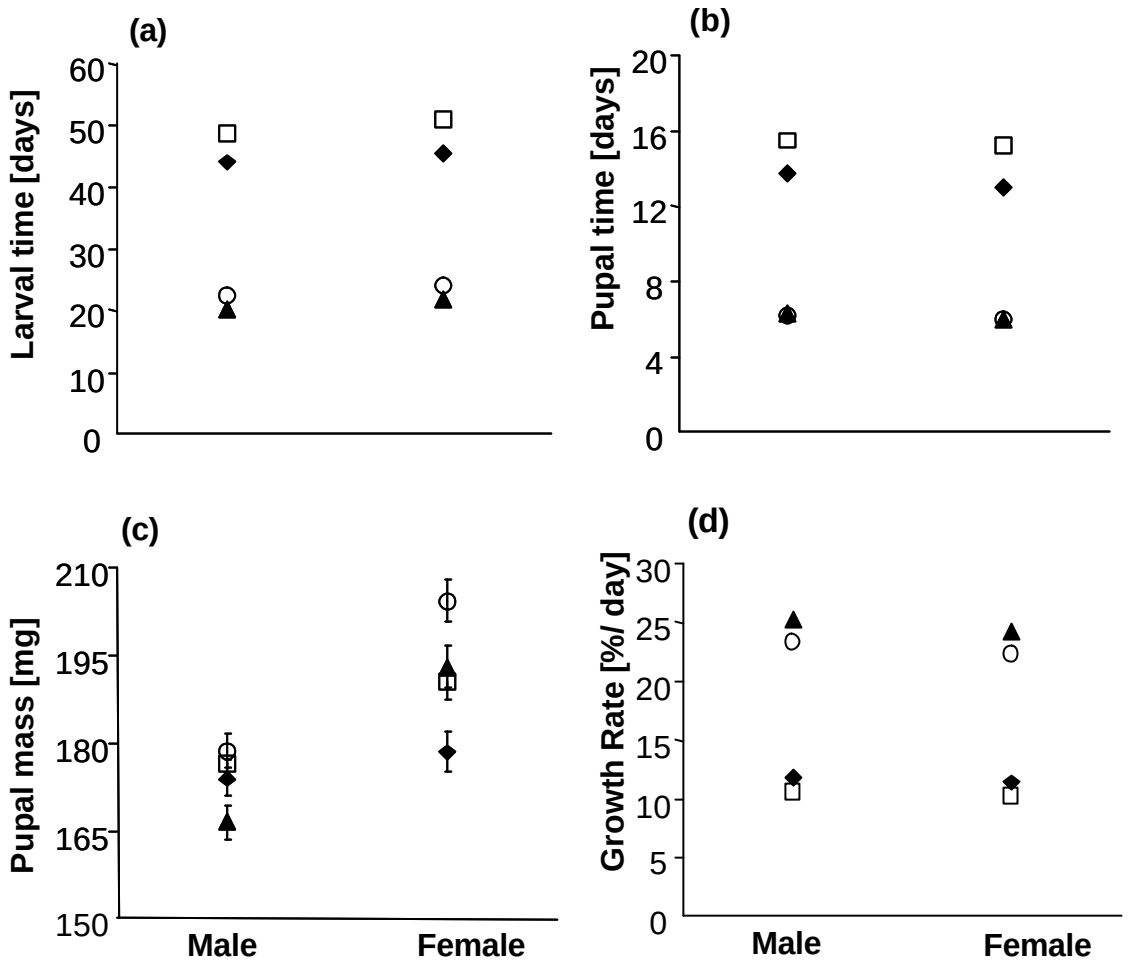

Figure 2. *Effects of parental and rearing temperature as well as sex on larval time (a), pupal time (b), pupal mass (c) and growth rate (d) in Bicyclus anynana. Squares: 20-20 (parental temperature 20°C/rearing temperature 20°C); diamonds: 27-20; circles: 20-27, triangles: 27-27. Open symbols: parental temperature: 20°C; filled symbols: parental temperature: 27°C. Standard error bars for data points are smaller than the symbols in some cases*

This developmental (and early adult) temperature effect persisted through to the second measurement, ten days after the division among oviposition temperatures (see also Fischer *et al*. 2003b). The effects of rearing temperature differed at least partly between the sexes. Rearing temperature by sex interactions were present for pupal, pupal time and larval growth rates.

In summary, this study shows that not only developmental and acclimation temperature affect life-history traits, but also the parental thermal environment. Such

effects may not only affect early development, but may also persist through to the adult stage. Furthermore this study shows that the thermal environment experienced in different generations and at different time points may interact in a complex manner, making any predictions about effect directions evidently difficult. Further, carry-over effects may yield antagonistic effects on different components of fitness, which may constrain the evolution of cross-generational adaptive plasticity.

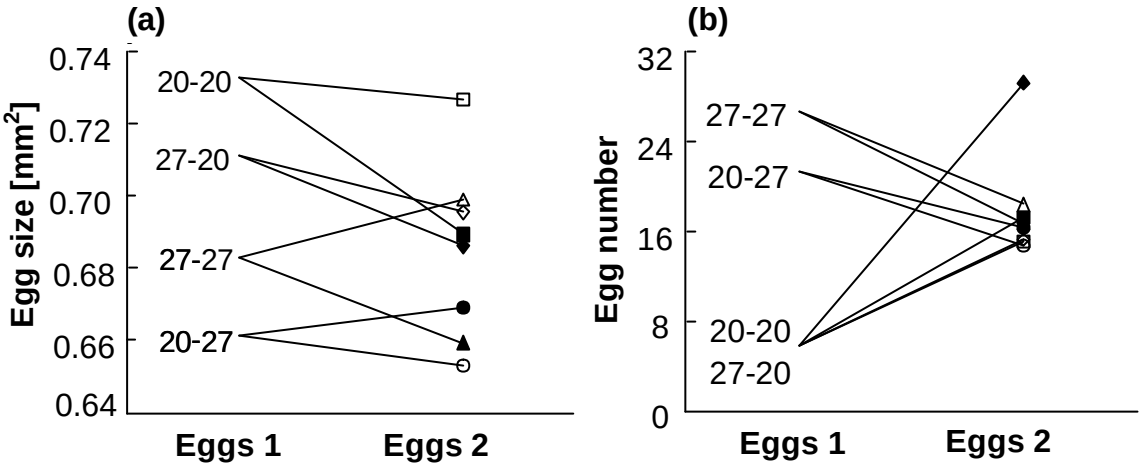

Figure 3. *Effects of parental, rearing and oviposition temperature on mean egg size (a) and number (b) in Bicyclus anynana. 20-20: parental temperature 20°C/rearing temperature 20°C; 20-27: parental temperature 20°C/rearing temperature 27°C and so on. Note that the 'rearing temperature' includes the first 6 days of adult life during which first eggs (Eggs 1) were laid, while later eggs (Eggs 2) were laid at the respective oviposition temperature where butterflies were kept from day 6 of adult life onwards. Open symbols: oviposition temperature 20°C; filled symbols: oviposition temperature 27°C. For better visibility no standard errors are given.*

2.3. Effects of the juvenile hormone mimic pyriproxyfen on female reproduction and longevity

In insects, hormones are the main regulators of life-history components like metamorphosis, behaviour, caste determination, diapause, polymorphisms and reproduction (Edwards *et al*. 1995; Gäde *et al*. 1997; Nijhout 1998; Flatt *et al.* 2005). The principle hormones influencing these components are the juvenile hormones (JHs henceforth) and the ecdysteroids. Given the anticipated importance of JH for egg development in this butterfly (cf. Ramaswamy *et al*. 1997) and the widespread

role of JH in mediating life-history plasticity (see above), I here examine the effects of a JH mimic (pyriproxyfen) on reproductive output in *B. anynana.*

When applied at the beginning of the oviposition period (days 4 or 5 of adult life), pyriproxyfen did affect reproductive output and longevity in female *B. anynana* butterflies. Earlier applications (days 0 and 2 of adult life), however, showed no effect on reproductive traits Figure 4). Likewise, applications on day 6 of adult life yielded weaker responses compared to applications on day 4 (Figure 4). These findings indicate that females are most sensitive to pyriproxyfen at the onset of oviposition, coinciding with naturally increasing JH titres and intensive vitellogenin synthesis in other Lepidoptera (e.g. Cusson *et al*. 1994; Ramaswamy *et al*. 1997; Zeng *et al*. 1997; Range *et al*. 2002).

Thus, provided that pyriproxyfen is applied during the sensitive period as outlined above, it consistently increased egg-laying rate and concomitantly fecundity in *B. anynana* females across experiments. The latter effect, however, was transient and restricted to a couple of days following the application (i.e. to the beginning of the oviposition period in the experiment; cf. Figure 4). This pattern matches the fact that effects of pyriproxyfen have a delay of around 24 hours only (Edwards *et al*. 1995), and that JH mimics degrade quite rapidly (Gilbert *et al*. 2000; Kamita *et al*. 2003). Comparable results were found in some other insects (fecundity in *Heliothis virescens*, Ramaswamy *et al*. 1997; ovarian mass in *Gryllus assimilis*, Zera *et al*. 1998; fecundity and egg-laying rate in *Nicrophorus* spp., Trumbo & Robinson 2004).

However, the increase in early fecundity was accompanied by a decrease in longevity throughout (cf. Herman & Tatar 2001; Tatar & Yin 2001; Trumbo & Robinson 2004). Empirical work, especially on *Drosophila*, has provided evidence that one cost of reproduction is acceleration of the rate of ageing (Barnes & Partridge 2003) and that JHs are involved in this process (Herman & Tatar 2001; Tatar *et al*. 2003; but see Richards *et al*. 2005).

In contrast to early fecundity, lifetime fecundity was not consistently higher in pyriproxyfen-treated females, but was even reduced compared to control females. Thus, when given enough time, control females may eventually outperform hormone-treated females (by maintaining higher oviposition rates late in life and by having a prolonged oviposition period). When differences in longevity are controlled for, the differences in lifetime fecundity across treatment groups disappear. Also in multiple regressions, longevity was the most important predictor of lifetime fecundity, followed by egg size.

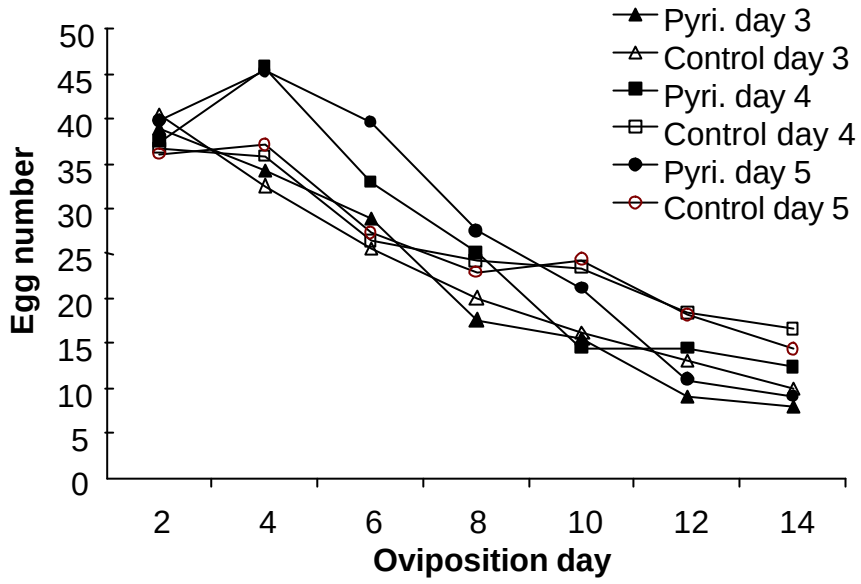

Figure 4. *Mean daily fecundity over time for groups of Bicyclus anynana females treated with 5 µg pyriproxyfen in 3 µl acetone on day 3, 4 or 5 following adult eclosion. Oviposition day 2 equals day 6 of adult life. Controls were treated with 3 µl pure acetone the same days. While pyriproxyfen and control groups do not differ on oviposition day 2, the former lay significantly more eggs than controls on days 4 to 6. From day 10 onwards, however, the pattern is reversed with controls laying significantly more eggs For clarity, no standard errors are given.*

In summary, the experiment showed that application of pyriproxyfen consistently increased early fecundity and egg-laying rate in *B. anynana* females. These increases were accompanied by a reduction in adult life span, thus potentially demonstrating a JH-mediated trade-off between present and future reproduction.

2.4. Ovarian dynamics, egg size and egg number in relation to temperature and mating status

One of the most striking and best-described phenomena with regard to variation in insect egg size is temperature-mediated plasticity (Azevedo *et al.* 1996; Crill *et al*. 1996; Yampolski & Scheiner 1996; Ernsting & Isaaks 1997; Blanckenhorn 2000; Atkinson *et al.* 2001; Fischer *et al*. 2003a, 2006b), but the potential mechanisms are unclear and described by multiple competing hypothesis (Wallin *et al*. 1992; Avelar 1993; Moore & Folt 1993; Huey *et al*. 1995; Van der Have & De Jong 1996; Van Voorhies 1996; Ernsting & Isaaks 1997; Fox & Czesak 2000; Blanckenhorn & Henseler 2005).

I investigated ovarian dynamics and reproductive output in relation to temperature and mating status in *Bicyclus anynana*, in order to unravel the hitherto unknown mechanisms underlying temperature-mediated plasticity in insect egg size using ovary staining and dissection techniques.

In line with results from previous studies, female *B. anynana* exhibited temperature-mediated plasticity in egg size and number, producing higher numbers of smaller eggs at the higher temperature, but fewer and larger eggs at the lower temperature (Fischer *et al.* 2003a,c). Regarding reproductive output, lifetime fecundity was roughly twice as high at 27 compared to 20°C (cf. Fischer *et al*. 2003a). This, however, does not reflect a trade-off between egg size and number, because variation in egg size is relatively small compared to that in egg numbers. Accordingly, total reproductive investment increased at the higher temperature (cf. Avelar 1993; Ernsting & Isaaks 1997, 2000; Fischer *et al*. 2003a). These findings challenge the idea of reduced costs of somatic maintenance at lower temperatures, enabling the allocation of more resources to reproduction and consequently larger egg sizes (Avalar 1993; Fox & Czesak 2000). The lack of differences in oocyte numbers across temperatures (see below) further suggests that reduced fecundity at lower temperatures is not caused by delayed oviposition (Wallin *et al*. 1992; Huey *et al*. 1995), as oocytes did not accumulate in the ovaries or oviducts in females ovipositing at the lower temperature. Regarding ovarian dynamics, the numbers of unchorionated oocytes dropped from an initial peak to lower levels during oviposition,

while numbers of chorionated oocytes were highest during oviposition days 0-6 (Figure 5). The percentage of chorionated oocytes increased during early oviposition due to chorionisation, followed by a later decline (Figure 5) (cf. Satyanarayana *et al*. 1991, 1992; Zeng *et al*. 1997; Delisle & Cusson 1999; Webb *et al*. 1999).

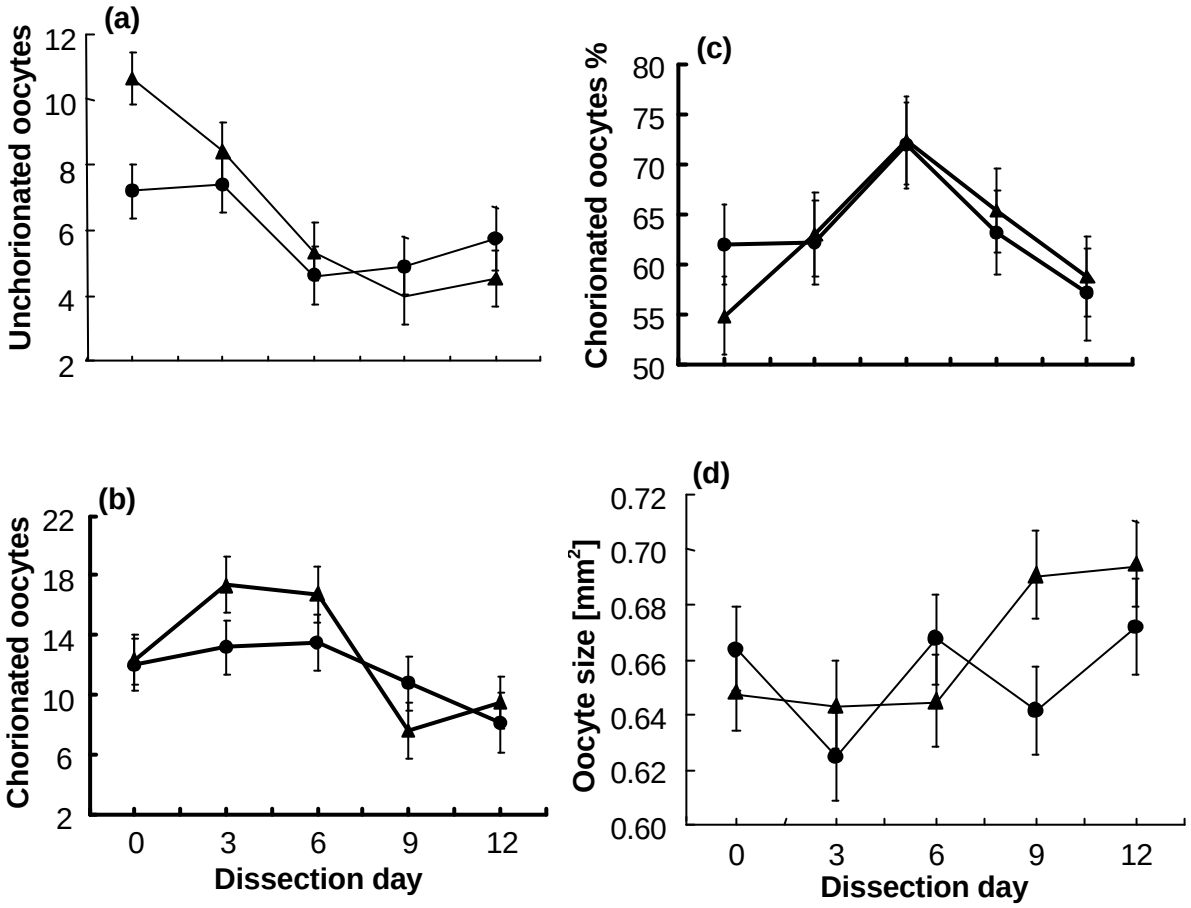

Figure 5. *Number of unchorionated oocytes (a), chorionated oocytes (b), percentage of chorionated oocytes (c), and oocyte size (d) over time in Bicyclus anynana females kept at 20°C or 27°C. Dissection day 2 equals day 6 of adult life. Given are means ± 1SEM. Triangles: 20°C, circles: 27°C.*

In contrast to these (expected) patterns over time, oocyte numbers did not differ between temperatures in both experiments (cf. Ernsting *et al*. 1992), while the size of terminal oocytes clearly increased at the lower temperature (Figure 5). Mating (and host-plant availability) had pronounced effects on egg development, reducing the number of chorionated and unchorionated oocytes, and decreasing the percentage of chorionated oocytes in mated compared to virgin females. These findings suggest that mating is not necessary to induce egg development, but that mating functions as

an important stimulus for egg deposition, without which virgin females retain their eggs as long as possible.

Interestingly, temperature effects on oocyte size were much more pronounced in mated than in virgin females, probably reflecting low levels of egg deposition in virgin females resulting in generally larger eggs (cf. Bauerfeind & Fischer 2005). Given equal numbers of oocytes in the ovaries across temperatures at any given time but much reduced egg laying rates at the lower temperature, the current data suggest reduced oocyte production (i.e. differentiation) rates at lower temperatures, as has been previously proposed (Van der Have & De Jong 1996; Ernsting & Isaaks 1997, 2000). As temperature generally slows down physiological processes in insects, resulting in reduced growth rates and extended development times, such reduced differentiation rates are likely to be accompanied by prolonged egg maturation times. Though both processes will jointly cause lower egg-laying rates (as observed), they do not *per se* affect egg size. If, however, oocyte growth (vitellogenesis) would be (even only marginally) less sensitive to temperature (Van der Have & De Jong 1996; Blanckenhorn & Henseler 2005) as compared to oocyte production rate and egg maturation time, both, reduced egg numbers and larger egg size would result at lower temperatures.

Consequently, my results support the notion of a differential temperature sensitivity of oocyte production (i.e. differentiation) versus vitellogenesis (i.e. growth), as postulated earlier by Van der Have & De Jong (1996) and Ernsting & Isaaks (1997, 2000). This hypothesis is based on a biophysical model. The biophysical model, supported here by empirical evidence, identifies temperature constraints on growth and differentiation. This, however, does not rule out that such physiological processes may have been exploited and thus further shaped by natural selection, resulting in overall adaptive egg sizes (Van der Have & De Jong 1996, Van Voorhies 1996; Ernsting & Isaacs 1997; Fischer *et al*. 2003; Blanckenhorn & Henseler 2005; Walters & Hassall 2006).

3

Summary (English and German)

3.1 Summary

As in nature organisms are often faced with variation in mean temperatures they have to adapt both plastically and genetically to these environmental conditions. As arthropod egg and thus progeny size is an evolutionary and ecologically significant trait, arthropod reproduction frequently shows temperature mediated phenotypic plasticity in egg size.

Using the tropical butterfly *Bicyclus anynana* as model organism, this study focuses on the mechanisms underlying temperature-mediated reproductive plasticity.

A half-sib experiment demonstrated that in *B. anynana* egg size responds in a plastic manner to oviposition temperature and that egg size is heritable with genetic variation in the plastic response to temperature. Additive genetic effects on egg size were weak however and differed quite substantially from other estimates of egg size heritability in *B. anynana.* The current estimate for additive genetic variance based on half-sibs is rather low. In contrast to sire effects, dam effects were highly significant throughout the present study, resulting in a relatively high dam component heritability. The most parsimonious explanation is that the maternal effects are due to female genotype.

These findings suggest that temperature-mediated egg size plasticity in this species is adaptive and that the conditions necessary for the evolution of phenotypic plasticity to occur are fulfilled.

Non-genetic parental effects can largely affect offspring phenotype and such effects are potentially adaptive. Maternal effects may also play a substantial role in the life history in *B. anynana.*

Using a temperature transfer experiment (chapter 5.2) I found substantial parental carry-over effects which were where most pronounced early in life and diminished over time. Higher developmental temperatures reduced development times and egg size, increased egg number, but did not affect pupal mass. Between-generation temperature effects influenced larval time, pupal time, larval growth rate and egg size analogously to developmental temperature, and additionally affected pupal mass but not egg number. Parental effects therefore seem to be important mediators of phenotypic plasticity in *Bicyclus anynana*, and partly yielded antagonistic effects on different components of fitness, which may constrain the evolution of cross-generational adaptive plasticity in this butterfly.

A likely candidate for the observed phenotypic plasticity in reproduction is juvenile hormone because of its widespread regulatory role in insect life-histories.

Female *Bicyclus anynana* butterflies given pyriproxyfen (chapter 6.1), a mimic of juvenile hormone, on day 4 or 5 of adult life, exhibited increased egg-laying rates and early fecundity, but reduced longevity compared to control animals. The effects of pyriproxyfen were transient and restricted to a couple of days after application. These findings indicate that females are most sensitive to pyriproxifen at the onset of oviposition, coinciding with naturally increasing juvenile hormone titres in other Lepidoptera and that juvenile hormone titres may be involved in a potential trade-off between present and future reproduction.

The reproductive mechanisms inside the ovaries are unknown however and multiple hypothesis try to explain this observed phenotypic plasticity in reproduction. A temperature transfer and ovary dissection experiment using female *B. anynana* (chapter 6.2) showed an increased reproductive investment with higher numbers of smaller eggs at the higher temperature, but fewer and larger eggs at the lower temperature. The number of unchorionated oocytes dropped from an initial peak to lower levels during oviposition, while numbers of chorionated oocytes were highest during oviposition days 0-6. The percentage of chorionated oocytes increased during early oviposition due to chorionisation, followed by a later decline. The number of oocytes did not differ between temperatures but the size of terminal oocytes clearly increased at the lower temperature. Mating reduced the number of chorionated and unchorionated oocytes, and decreased the percentage of chorionated oocytes suggesting that mating is an important stimulus for egg deposition, without which virgin females retain their eggs as long as possible.

Given equal numbers of oocytes in the ovaries across temperatures at any given time but much reduced egg laying rates at the lower temperature, the data suggest reduced oocyte production (i.e. differentiation) rates at lower temperatures.

3.2 Zusammenfassung

Da Lebewesen in der Natur oft mit Temperatur variation konfrontiert werden, sind sie gezwungen, sich entweder plastisch oder genetisch an diese wechselnden Umweltbedingungen anzupassen. Weil die Eigröße von Arthropoden, und damit auch die Größe ihrer Nachkommen, aus evolutionärer sowie ökologischer Sicht eine wichtige Eigenschaft darstellt, zeigt die Fortpflanzung von Arthropoden häufig eine durch die Temperatur vermittelte phänotypische Plastizität.

Die vorliegende Studie, in welcher der tropische Schmetterling *Bicyclus anynana* als Modellorganismus verwendet wird, konzentriert sich auf die Mechanismen, welche der temperaturvermittelten reproduktiven Plastizität zu Grunde liegen.

Ein Halbgeschwister-Verpaarungsexperiment (Kapitel 5.2) zeigte, dass die Eigröße von *B. anynana* in Abhängigkeit von der Temperatur schwankt. Weiterhin ergab sich, dass die Plastizität von Eigröße eine erbliche Komponente aufweist. Die additiven genetischen Effekte auf die Eigröße waren jedoch schwach ausgeprägt und unterschieden sich ziemlich stark von anderen Studien, welche sich der Eigrößenerblichkeit von *Bicyclus anynana* widmeten.

Im Gegensatz zur additiven genetischen Varianz waren die mütterlichen Effekte in dieser Untersuchung durchgehend hochsignifikant, was eine relativ hohe Erblichkeit der mütterlichen Anteile zur Folge hatte. Die naheliegendste Erklärung ist, dass die maternalen Effekte vom weiblichen Genotyp stammen.

Diese Resultate implizieren, dass die durch die Temperatur vermittelte Eigrößenplastizität bei dieser Art adaptiv sein könnte, da die notwendigen Bedingungen für das Auftreten evolutiver Veränderungen von phänotypischer Plastizität erfüllt sind.

Nicht-genetische parentale Effekte können den Phänotyp ebenfalls stark beeinflussen und sind möglicherweise adaptiv. Maternale Effekte könnten in der Lebensgeschichte von *B. anynana* eine erhebliche Rolle spielen. Mit Hilfe eines Temperaturtransfer-Experimentes (Kapitel 5.2) wurden beträchtliche generationenübergreifende, parentale Effekte festgestellt, welche in der Frühentwicklung am stärksten ausgeprägt waren und sich mit der Zeit verringerten. Höhere Temperaturen während der Entwicklung verminderten Entwicklungszeiten und Eigröße und ließen die Eizahl ansteigen, wirkten sich jedoch nicht auf das Puppengewicht aus. Die Temperaturbedingungen in der vorhergehenden Generation beeinflussten, ähnlich der Entwicklungstemperatur, die Entwicklungszeit der Puppen

und Larven, die larvale Wachstumsrate sowie die Eigröße. Darüberhinaus wirkten sie sich auf das Gewicht der Puppen aus, nicht jedoch auf die Eizahl. Parentale Effekte sind daher wichtige Vermittler der phänotypischen Plastizität von *B anynana* und können antagonistisch auf verschiedene Fitnesskomponenten wirken, welche die Evolution der generationenübergreifenden adaptiven Plastizität bei dieser Schmetterlingsart einschränken könnten.

Derartige phänotypische Plastizität könnte auf Variation in Juvenilhormon-Titern zurückzuführen sein, vor allem wegen seiner weitverbreiteten regulatorischen Rolle in der Lebensgeschichte von Insekten.

Weibliche *B. anynana*-Schmetterlinge, denen am 4. oder 5. Tag ihres adulten Lebens Pyriproxifen (Kapitel 6.1), ein Analog von Juvenilhormon, verabreicht wurde, wiesen, verglichen mit den Kontrolltieren, gesteigerte Eiablageraten bei verkürzter Lebensdauer auf. Die Auswirkungen von Pyriproxifen waren vorübergehend und auf einige Tage nach der Anwendung beschränkt. Diese Ergebnisse deuten darauf hin, dass Weibchen zu Beginn ihrer Eiablageperiode am sensibelsten auf Pyriproxifen reagieren. Dies stimmt mit der Tatsache überein, dass der Juvenilhormontiter bei anderen Lepidopteren in dieser Zeit von Natur aus ansteigt. Weiterhin könnte durch Juvenilhormon ein möglicher ‚Trade-off' zwischen gegenwärtiger und zukünftiger Fortpflanzung gesteuert werden.

Effekte der Temperatur auf die Eireifung wurden bislang wenig untersucht, wobei verschiedene alternative Hypothesen versuchen, phänotypische Plastizität bei der Fortpflanzung zu erklären. Ein Temperaturtransfer-Experiment mit anschließender Ovarienpräparierung bei *B. anynana*-Weibchen (Kapitel 6.2) ergab einen gesteigerten Fortpflanzungsaufwand mit einer höheren Anzahl kleinerer Eier bei der höheren Temperatur und einer kleineren Anzahl größerer Eier bei der niedrigeren Temperatur. Die Anzahl unchorionisierter Oozyten fällt während der Eiablage von einem anfänglichen Höhepunkt zu einer geringeren Zahl hin ab, wobei die Anzahl chorionisierter Oozyten während den Tagen 0-6 der Eiablage am höchsten war. Der prozentuale Anteil stieg zu Beginn der Eiablage aufgrund von Chorionisierung, gefolgt von einem späteren Rückgang. Die Zahl der Oozyten unterschied sich nicht zwischen den Temperaturen, aber die Größe der terminalen Oozyten stieg bei niedrigerer Temperatur eindeutig an.

Paarung minderte die Anzahl chorionisierter und unchorionisierter Oozyten. Dies lässt darauf schließen, dass die Paarung einen wichtigen Stimulus für die Eiablage

darstellt, ohne den unverpaarte Weibchen ihre Eier so lange wie möglich zurückbehalten.

Angesichts gleichbleibender Oozytenzahlen in den Ovarien über alle Temperaturen zu jedem Zeitpunkt, aber stark verminderter Eiablageraten bei niedrigeren Temperaturen, deuten die Daten auf verminderte Raten der Oozytenproduktion bzw. Differenzierung bei niedrigeren Temperaturen hin.

4

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5

The genetic background of temperature mediated reproductive plasticity

5.1 The evolutionary genetics of egg size plasticity in a butterfly

Marc J. Steigenga*[1], Bas J. Zwaan[2], Paul M. Brakefield[2] & Klaus Fischer[1]

[1]Department of Animal Ecology I, University of Bayreuth, D-95440 Bayreuth, Germany

[2]Institute of Biology, Leiden University, P.O. Box 9516, NL-2300 RA Leiden, The Netherlands

***Author for correspondence:**

Marc Steigenga
Department of Animal Ecology I
Bayreuth University
P.O. Box 101 251
D-95440 Bayreuth, Germany

Tel.: +49-921-553079
Fax: +49-921-552784
E-mail: marc.steigenga@uni-bayreuth.de

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Abstract

The evolution of phenotypic plasticity requires that it is adaptive, genetically determined, and exhibits sufficient genetic variation. For the tropical butterfly *Bicyclus anynana* there is evidence that temperature-mediated plasticity in egg size is an adaptation to predictable seasonal change. We here set out to investigate heritability in egg size and genetic variation in the plastic response to temperature in this species, using a half-sib breeding design. Egg size of individual females was first measured at a high temperature 4 days after eclosion. Females were then transferred to a low temperature and egg size was measured after acclimation periods of 6 and 12 days, respectively. Overall, additive genetic variance explained only 3 to 11 % of the total phenotypic variance, whereas maternal effects were more pronounced. Genotype-environment interactions and cross-environmental correlations of less than unity suggest that there is potential for short-term evolutionary change. Our findings strengthen the support for the adaptive nature of temperature-mediated plasticity in egg size.

Introduction

One of the central goals of evolutionary biology is to understand the processes by which organisms adapt to complex environments. To detect such processes, the causes and the effects of variation in traits that influence fitness need to be known. Two sources of phenotypic variation in life-history traits have long been recognized, namely genetic differentiation and effects of different environments on the expression of the phenotype (Schmalhausen 1949; Endler 1986). The latter source of variation, called phenotypic plasticity, may be merely a biochemical or physiological interaction of the organism with its environment, or it may be an adaptation to spatially heterogeneous or temporarily varying environments (Levins 1963; Bradshaw 1965). An adaptive hypothesis requires that phenotypic changes enhance the performance of an individual organism in the environment in which those changes were induced (Schmalhausen 1949; Stearns 1989). Recently, advances in evolutionary physiology have stimulated a renewed and increasing interest in the magnitude and nature of non-genetic effects on the expression of an organism's phenotype (e.g. Nylin & Gotthard 1998; Feder *et al.* 2000; Pigliucci 2003).

As for any other biological feature, the evolution of phenotypic plasticity requires that (1) it is adaptive, (2) it is at least in part genetically determined, and (3) it exhibits sufficient standing genetic variation in the population concerned (cf. Ernande *et al.* 2004). The adaptive nature of phenotypic plasticity is usually assessed by its fitness consequences in different environments. The genetic basis is evaluated by means of heritability estimates and the proportion of phenotypic variance explained by genetic factors. Genetic variation in phenotypic plasticity, finally, is explored by the genotype-environment interaction, through quantifying the variation in the plastic response to environmental change among genotypes (Scheiner & Lyman 1991; Stearns 1992; Falconer & Mackay 1996). In summary, the study of phenotypic plasticity is quite a challenging enterprise, and consequently there are only a few studies testing experimentally for all three conditions in animals (e.g. Czesak & Fox 2003; Ernande *et al.* 2004). Currently, the most serious lack in our understanding of phenotypic plasticity is experimental studies on its genetic basis (Scheiner & Lyman 1991; Scheiner 1993).

Here, we set out to test for the existence of genetic variation in the plastic response of egg size to temperature in the tropical butterfly *Bicyclus anynana* (Butler 1879), employing a half-sib breeding design (e.g. Falconer & Mackay 1996). For several reasons we believe that our study system is highly appropriate for studying the genetics of phenotypic plasticity. *First*, egg size is generally believed to be closely related to fitness (Fox & Czesak 2000). *Second*, egg size is known to readily respond to differences in developmental and adult temperature in *B. anynana*, with lower temperatures causing larger egg sizes and vice versa (Fischer *et al.* 2003a,b,c, 2004). This pattern is also observed in many other arthropods (e.g. Azevedo *et al.* 1996; Blanckenhorn 2000; Atkinson *et al.* 2001). *Third*, temperature is considered one of the most important mediators of phenotypic variation in ectothermic animals, resulting in not only predictable changes in egg size, but also in body size (temperature-size rule; e.g. Atkinson 1994; Partridge & French 1996; Chown & Gaston 1999). *Fourth* and perhaps most importantly, there is evidence that temperature-related egg size plasticity in *B. anynana* is adaptive (Fischer *et al.* 2003a,c).

Recent studies showed that *B. anynana* females kept at a lower oviposition temperature laid larger but fewer eggs than those kept at a higher temperature. Based on differential survival probabilities among temperatures, these data suggest that it does pay off to produce fewer but larger offspring (with increased fitness) at a lower temperature, but more and smaller offspring at a higher temperature where offspring survival is generally high in this tropical butterfly (Fischer *et al.* 2003a,c). Consequently, females should be able to maximize their reproductive success by adjusting the size of eggs to the temperature experienced during oviposition, which could provide a predictable cue for the environmental conditions experienced by the offspring in early life. These suppositions match closely environmental demands, as *B. anynana* lives in a seasonal environment with a beneficial wet season of high temperatures, and a rather adverse dry season when average temperature is low (Brakefield 1997). Thus, those results can be readily interpreted within an ecologically realistic context.

If this scenario about the adaptive matching of variation in egg size with seasonal temperature environments holds, genetic variation in the plastic response to temperature for natural selection to act upon is expected (as long as the variation has not been completely eroded; Via & Lande 1985; Scheiner 1993). The existence of a genotype-environment interaction would be indicative of the potential for short term evolutionary change, and thus that natural selection should be able to reshape the plastic response to temperature, for example in response to a changing relationship between temperature and ecological seasons due to climate change (see Roskam & Brakefield 1999). However, an earlier study failed to find evidence of genotype-environment interaction when focussing on temperature environments during pre-adult development (Fischer *et al.* 2004). Here, in contrast, we deal with plasticity in the adult stage that is probably more relevant to environments in the wild (Fischer *et al.* 2003a,b,c, 2004), and we also employ an experimental design with more power.

Materials and Methods

Study organism

Bicyclus anynana is a tropical, fruit-feeding butterfly with a distribution ranging from Southern Africa to Ethiopia (Larsen 1991). The species exhibits striking phenotypic plasticity (two seasonal morphs) that is thought to function as an adaptation to

alternative wet-dry seasonal environments and the associated changes in resting background and predation (Brakefield 1997; Lyytinen *et al.* 2004). A laboratory stock population of *B. anynana* was established at Leiden University in 1988 from about 80 gravid females caught at a single locality in Malawi. Several hundred adults are reared in each generation, maintaining high levels of heterozygosity (Saccheri & Bruford 1993). For this study, butterflies from the stock population were used.

Experimental design

A half-sib breeding design was used to estimate additive genetic variation in egg size and to explore genetic variation in temperature reaction norms (Falconer & Mackay 1996). All butterflies were reared in a climate room at 27°C, high humidity, and a photoperiod of L12:D12. Parental generation larvae were fed on young maize plants in population cages (50 x 50 x 80 cm). The resulting pupae were collected from the plants and transferred to cylindrical hanging cages. Following adult eclosion, males and females were individually marked and kept separated by sex until mating. Mating trials started as soon as there were sufficient females (they eclose a few days later than males due to protandry; Zijlstra *et al*. 2002; Fischer *et al.* 2003a). To keep males in fresh condition for later matings, they were kept in another climate room at 20°C (high humidity and L12:D12 throughout; male larval or adult temperature does not affect female egg size; Fischer *et al.* 2003b).

For mating, equal numbers of males and females were put together in hanging cages for 2-3 hours, where they were continuously observed. Mating pairs were transferred individually to 1 L plastic containers with a small egg-laying plant. After mating males (sires) were removed from the containers and were allowed to recover for one day, after which they were set up for mating again. Sires that failed to mate in the first round were discarded, but unmated females were re-used. This lead to a total of 34 sires mated successfully to an average of 2.3 females (dams) (range 2-4), creating a total of 78 full-sib families. These numbers exclude all sires having mated with less than two females as well as all dams producing less than three female offspring. To create full-sib families, dams were allowed to oviposit individually for one week, after which the eggs were transferred to elongated, sleeve-like cages containing a young maize plant for further development. Afterwards, an additional group of eggs from each dam was collected to measure egg size (see below).

Female F_1 pupae were weighed on the day following pupation to the nearest 0.01 mg and afterwards kept individually until adult eclosion, while all male pupae were pooled in a single hanging cage. After adult eclosion, females were marked individually and kept separated from males for one day. Then, females were given two days for mating with random males, after which they were set up individually for egg laying in 1 L plastic containers with a small oviposition plant. Eggs were collected and measured the next day (i.e. on day four of adult life). These eggs were the first ones laid within the females' adult life span, thus effectively controlling for any confounding effects of female age (e.g. Karlsson & Wiklund 1984; Brakefield *et al.* 1994; Braby & Jones 1995).

Having collected the initial eggs, individual females were transferred from 27°C to 20°C to induce a plastic response in egg size. Further eggs were collected and measured after acclimation periods of 6 and 12 days, respectively (i.e. on days 10 and 16 of adult life). In *B. anynana* a clear plastic response to temperature can be expected to occur within 6-10 days (Fischer *et al.* 2003a,b,c). We have chosen to transfer females from high to low temperature (and not vice versa) because the plastic response appears to be more pronounced in the cold as compared to the warmth (Fischer *et al.* 2003a,c), and more importantly because the pattern of reduced egg size at warmer temperatures is potentially confounded by effects of female age. As in most other arthropods, egg size declines with female age in *B. anynana* (Wiklund & Karlsson 1984; Fox & Czesak 2000; for *B. anynana* see Brakefield *et al.* 1994; Fischer *et al.* 2003a,b). In total, 545 females (involving on average 7 female offspring per dam) laid eggs for analysis. Throughout all experiments, butterflies had access to moist banana (replaced every other day) for adult feeding.

Egg measurements

As the eggs of *B. anynana* are nearly perfect spheres, egg size was measured as cross-sectional area (mm^2) using a digital camera (Leica DC200) connected to a binocular microscope. The resulting images were analysed using Scion Image public software (Scion Corporation, 2000). Tight correlations between egg area (applying image analysis) and egg mass as well as hatchling size confirm that this method

provides a highly reliable measurement of egg size in *B. anynana* (Fischer *et al.* 2002). To calculate egg size for individual females, the mean of about 10 eggs was used. Previous experiments showed that the means did not change substantially above a critical minimum number of 7-8 eggs (data not shown).

Statistical analyses

In a first step, effects of sire, dam (nested within sire) and time (i.e. comparing the measurements from days 4, 10 and 16 of female adult life) were analysed. As there are three measurements per individual female, a repeated measurements analysis of variance (ANOVA) was used. Here a significant time effect indicates a change in egg size over time, and interactions between sire respectively dam and time would indicate genotype-environment interactions (note that repeated measures ANOVAs do not support random factors; thus sire and dam are treated as fixed effects). Thereafter, we applied ANOVAs with sire and dam (nested within sire) as random factors separately to the different egg measurement days and to pupal mass, using estimated mean squares (EMS). Additive genetic variances (V_A) in egg size (separately for the different measurement days) and pupal mass were calculated from variance components using the restricted error maximum likelihood method (REML). Additive genetic variances were estimated according to Falconer & Mackay (1996) as 4 V_s (V_s is the among sire component).

As in other studies, maternal effects variance (V_M) was calculated by assuming that dominance variance and epistatic interactions were zero (such that $V_M = V_d - V_A/4$, where V_d is the dam variance; Fox *et al.* 1999; Czesak & Fox 2003). This does not mean that dominance and epistatic interactions do not occur, but rather that these are included in the maternal effects variance, as a traditional half-sib design does not allow for a distinction between these sources of variation. Thus, these estimates set an upper limit to the maternal effects. Sire and dam component heritabilities were computed following Falconer & Mackay (1996) as 4 V_s / V_P and 4 V_d / V_P (V_P is the total phenotypic variance), respectively. Additionally, heritability of egg size was calculated by means of parent-offspring regression (i.e. by regressing the mean egg size of all full-sib sisters from the first measurement made at high temperature on day 4, weighted by the number of sisters in each full-sib family, on the mean egg size of the mother, also at high temperature).

To investigate the evolutionary potential of phenotypic plasticity in egg size we calculated genetic cross-environmental correlations of half-sib family means (Via & Lande 1985; Via 1993), using Pearson's product moment correlation. As this estimate is potentially confounded by within-family variance in the (co)variance terms, we additionally applied a mixed-model ANOVA (Fry 1992) as was done previously for this system (Wijngaarden *et al.* 2002). In short, a mixed-model ANOVA (with half-sib family and temperature as random and fixed effects, respectively) was used with the variance component for the family effect providing an estimate of the family covariance across temperatures. Additionally, the variance components of the half-sib family effect within each temperature were estimated. The restricted error maximum likelihood (REML) method was used to estimate variance components. The cross-environment genetic correlation was then estimated as the family covariance across temperatures divided by the geometric mean family variance in each temperature environment. This method provides reliable and robust estimates (Windig 1997). Confidence intervals for correlation coefficients were obtained using the z-transformation as described in Sokal & Rohlf (1995). Throughout, means are given ± 1 standard error. All statistical tests were performed using JMP version 4.02.

Results

Egg size plasticity in relation to temperature

Egg size showed a clear plastic response to oviposition temperature, increasing after the transfer from 27°C to 20°C from initially 0.649 ± 0.002 mm^2 on day 4 to 0.665 ± 0.002 and 0.705 ± 0.002 mm^2 on days 10 and 16 of female adult life, respectively. Those increases in area are equivalent to increases of 5.3 % (day 10) and 15.4 % (day 16) in egg volume. A repeated measurements ANOVA on days 4, 10, 16 confirmed significant effects of sire ($F_{23,388}$ = 2.1, P = 0.0025), dam (nested within sire; $F_{45,388}$ = 2.1, P < 0.0001) and time ($F_{2,387}$ = 235.7, P < 0.0001). The interaction between time and dam was also significant ($F_{90,774}$ = 1.7, P = 0.0003; Figure 1a), however, the one between time and sire was marginally non-significant ($F_{46,774}$ = 1.3, P = 0.068; Figure 1b). By including all females measured (i.e. including those from groups with small sample sizes; see Methods), the time-sire interaction reaches significance ($F_{66,854}$ = 1.6, P = 0.0042). These interactions suggest the existence of genetic variation in reaction norms. Cross-environmental genetic

correlations were positive and significant, explaining 35-52 % of variance (Table 1). However, the 95 % confidence intervals consistently excluded unity, suggesting that the correlations differ significantly from unity. Both methods, product-moment correlations and mixed-model ANOVAs, gave comparable results with overlapping confidence intervals throughout (Table 1).

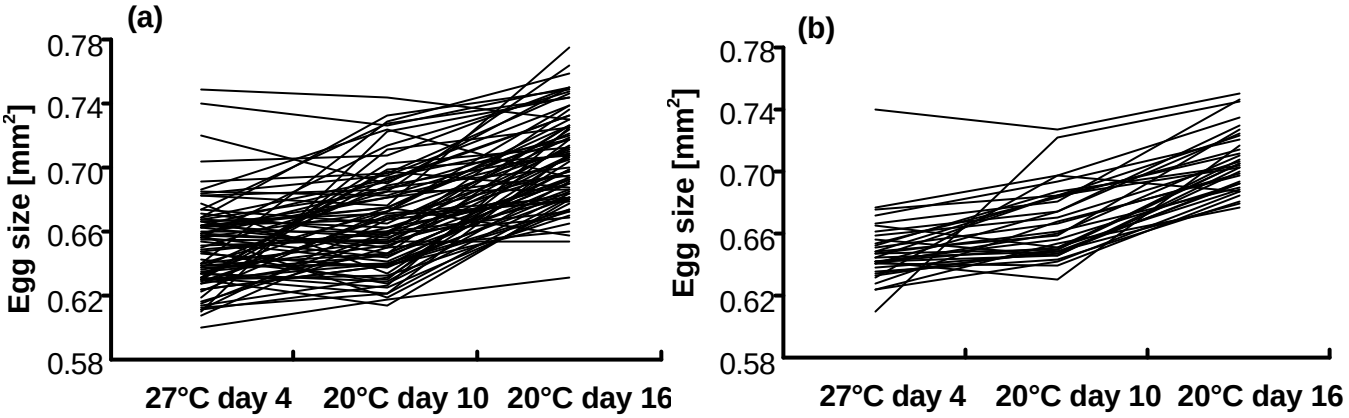

Figure 1. *Reaction norms for egg size in relation to adult temperature in the butterfly Bicyclus anynana. Each line represents the mean values of a full-sib (a) or half-sib family (b), respectively. Measurements took place on days 4, 10 and 16 of the females' adult life span. Females were transferred from 27 to 20°C on day* 4.

Heritability of egg size

In spite of the prevalence of a significant sire effect when the data from all three measurement days were jointly analysed (see above), separate analyses for each of those days failed to reveal significant sire effects (Table 2). This difference results from the fact that in the latter analysis the dams-within-sire mean square is the denominator for the *F*-ratio of the sire effect. Overall, additive genetic effects on egg size were weak, as judged by the low estimates for additive genetic variance (V_A), explaining 3 to 11 % of the total phenotypic variance (Tables 2 and 3). Dam effects, in contrast, were highly significant throughout and maternal effect variance (V_M) explained 10 to 14 % of the total phenotypic variance (Table 2). Accordingly, the dam component heritability was relatively high (h^2 0.45 to 0.62; Table 3). These results suggest the existence of either non-additive genetic variances or rather strong effects of the female's environment, phenotype or genotype on egg size.

Table 1. *Genetic cross-environmental correlations for egg size at different temperatures. Measurements took place on days 4, 10 and 16 of the females' adult life span. Females were transferred from 27 to 20°C on day 4. The correlations between measurement days 10 and 16 was included because females had only begun to respond to the temperature change by day 10. CI: 95 % confidence interval. a) Calculations based on product-moment correlations of half-sib family means weighted by the number of females. b) Calculations based on mixed-model ANOVAs (see Methods)*.

a)

	r	**CI**	***t***	***P***	***n***
Day 4 (27°C) – Day 10 (20°C)	0.676	0.608 - 0.744	21.08	< 0.0001	573
Day 4 (27°C) – Day 16 (20°C)	0.591	0.516 - 0.666	17.56	< 0.0001	522
Day 10 (20°C) – Day 16 (20°C)	0.687	0.616 - 0.758	21.66	< 0.0001	515

b)

	r	**CI**
Day 4 (27°C) – Day 10 (20°C)	0.680	0.613 - 0.747
Day 4 (27°C) – Day 16 (20°C)	0.654	0.585 - 0.722
Day 10 (20°C) – Day 16 (20°C)	0.722	0.653 - 0.790

Because a female's egg size may covary with her body mass (for butterflies e.g. Garcia-Barros, 2000), genetic or maternal effects on egg size could be largely due to variation in body size. In our study, however, egg size was only weakly related to pupal mass on egg measurement days 4 and 16, and not at all on day 10 (day 4: r = 0.21, P < 0.0001, n = 600; day 10: r = 0.05, P = 0.25, n = 576; day 16: r = 0.11, P = 0.009, n = 527). Repeating all analyses presented in tables 2 and 3, with the inclusion of pupal mass as a covariate to the model, does not change any of the results qualitatively (results not shown). Additive genetic variance was then estimated to explain 6 to 8 % of the total phenotypic variance, with maternal effect variance accounting for 8 to 14 %. The estimate for heritability of egg size derived from the parent-offspring regression was h^2 = 0.20 ± 0.04 (r = 0.20, slope = 0.10, P < 0.0001, $n_{families}$ = 78, $n_{individuals}$ = 517).

Heritability of pupal mass

Pupal mass was significantly affected by both sire and dam effects (Table 2). Additive genetic variance explained 25 % of the total phenotypic variance in pupal mass (Tables 2 and 3).

Table 2. *Nested analysis of variance (using EMS) and variance component analysis (using REML) for egg size (at different temperatures) and pupal mass in the butterfly Bicyclus anynana. Egg measurements took place on days 4, 10 and 16 of the females' adult life span. Females were transferred from 27 to 20°C on day 4. V_s, among-sire variance component; V_d, among-dam variance component ; V_e, error variance; V_p, total phenotypic variance; V_A, additive genetic variance; V_M, maternal effects variance; V_E, environmental variance.*

	Analysis of variance					Genetic	
		MS			Observational variance component	Variance Component	Proportion of V_p explained
Source	**df**	**(x 10^{-3})**	***F***	***P***	**(x 10^{-4})**	**(x 10^{-3})**	**± SE (%)**
Day 4 (27°C)							
Sire	23	3.30	0.82	0.6944	V_s = 0.178	V_A = 0.0712	2.9 ± 11.1
Dam (Sire)	45	4.08	1.89	0.0007	V_d = 2.711	V_M = 0.253	10.4 ± 2.0
Error	471	2.17			V_e = 21.458	V_E = 2.11	86.7
r^2 = 0.23					V_p = 24.347		
Day 10 (20°C)							
Sire	23	5.21	1.12	0.3642	V_s = 0.734	V_A = 0.294	10.8 ± 16.0
Dam (Sire)	45	4.68	2.10	0.0001	V_d = 3.862	V_M = 0.313	11.5 ± 2.1
Error	449	2.27			V_e = 22.646	V_E = 2.12	77.8
r^2 = 0.28					V_p = 27.241		
Day 16 (20°C)							
Sire	23	3.28	0.68	0.8361	V_s = 0.381	V_A = 0.152	5.7 ± 14.6
Dam (Sire)	45	4.83	2.18	< 0.0001	V_d = 4.159	V_M = 0.378	14.2 ± 2.5
Error	403	8.94			V_e = 22.121	V_E = 2.14	80.3
r^2 = 0.29					V_p = 26.661		
Pupal Mass							
Sire	23	166371	1.90	0.0318	V_s = 340564.81	V_A = 136226	25.1 ± 17.0
Dam (Sire)	45	890247	1.96	0.0003	V_d = 400050.84	V_M = 5949	1.1 ± 0.5
Error	474	454400			V_e = 4689420.40	V_E = 400829	73.8
r^2 = 0.25					V_p = 5430036.05		

Maternal effects variance, in contrast, explained very little of the phenotypic variation in pupal mass (Table 2). Thus, the mother's environment, phenotype or genotype did not substantially influence pupal mass. Consequently, sire and dam component heritabilities were rather similar and can be regarded as equally reliable, and therefore their combination based on the resemblance between full sibs may be taken as the best estimate (h^2 = 0.27; sire + dam in Table 3; see Falconer & Mackay, 1996).

Table 3. *Estimates for sire and dam component heritability as well as for the heritability of both components combined (± 1 SE) of egg size at different measurement days and pupal mass.*

	Egg size			Pupal mass
	Day 4 (27°C)	**Day 10 (20°C)**	**Day 16 (20°C)**	
Sire	0.029 (0.11)	0.108 (0.16)	0.057 (0.15)	0.251 (0.17)
Dam	0.112 (0.19)	0.567 (0.24)	0.624 (0.24)	0.291 (0.19)
Sire + Dam	0.239 (0.02)	0.337 (0.20)	0.341 (0.19)	0.273 (0.18)

Discussion

Plasticity in egg size

In agreement with earlier studies using *B. anynana* as a model organism (Fischer *et al.* 2003a,b,c, 2004), temperature clearly induced a plastic response in egg size. As all females were reared in a common environment, the differences found cannot be attributed either to temperature-mediated effects on body size or to physiology during development, but are a direct consequence of differences in the oviposition environment. Note that, in agreement with most other arthropods, butterfly egg size usually declines with female age (there is not a single exception known for Lepidoptera; Fox & Czesak 2000), making the increase in egg size even more striking than is apparent from the values given above. Such temperature-related plasticity, resulting in larger offspring at lower temperatures, seems to be a near universal rule in ectotherms (Yampolski & Scheiner 1996; Fox & Czesak 2000; Atkinson *et al.* 2003). The interactions between time (i.e. acclimation period) and sire respectively dam suggest the existence of genetic variation in the plastic response to temperature, and thus the potential for short-term evolutionary change. This genotype-environment interaction is graphically evident in crossing of the

temperature reaction norms in Figure 1. While most of the full-sib families follow the expected pattern of an increase in egg size with time, some families behave differently in showing virtually no response (between -2 and +2 % change in egg volume between first and last egg measurement only, n = 7) or even, in a single case, a decrease in egg size (> - 2 %). However, it is important to note that the time-sire interaction is of borderline significance and caused by only a small proportion of the families implying only rather weak evidence for the existence of a genotype-environment interaction.

A similar pattern was found in an earlier study, though in that case genotype-environment interactions were overall not significant (Fischer *et al.* 2004). The lack of statistical support there was probably due to insufficient statistical power. In addition to the genetic variation in reaction norms, cross-environmental correlations, although being positive, were significantly less than unity suggesting that any constraints on evolutionary potential due to genetic correlations are unlikely to prevent evolution to new phenotypic values in different environments although they may reduce the rate of such evolution (Beldade *et al*. 2002; Zijlstra *et al*. 2004). The correlations explain about 40-50 % of the total variance, thus leaving scope for evolutionary change in different directions and strengthening the case for genetic variation in plasticity.

Heritability of egg size

In spite of a significant sire effect when the data from all three measurement days were jointly analysed using a repeated measurements ANOVA, separate analyses for each of these days failed to reveal significant sire effects. These results suggest that, overall, additive genetic effects on egg size were weak (estimates for V_A range between 3 to 11 %). These results differ quite substantially from other estimates of egg size heritability in *B. anynana*. Parent-offspring regressions gave estimates of 0.2 (this study) and ca. 0.4 (Fischer *et al.* 2004), and a full-sib design showed a broad-sense heritability of ca. 0.2 (Fischer *et al.* 2004). Realized heritability, as revealed by selection experiments, was also about 0.4 (Fischer *et al.* in prep.). Thus compared to those other results the current estimate for additive genetic variance based on half-sibs is rather low. Although we cannot explain this difference (note e.g. the rather low broad-sense heritability), some of the variation may arise from selecting on maternal effects and/or specific (rare) genotypes during selection experiments thus

subsequently increasing realized heritability. Also the parent-offspring regressions include maternal effects, with the data based on the half-sib analysis giving the only unbiased estimate of additive genetic variance. At this stage, we conclude that heritability of egg size in *B. anynana* is low to moderate, as expected for a typical life-history trait (Roff 1997).

Generally, there are surprisingly few data available on genetic variation in egg size within insect populations (Fox & Czesak 2000). In two seed beetles, egg size was found to be highly heritable and estimates ranged between 0.22 and 0.91, varying between populations and host plants (Fox *et al.* 1999). For one of those species recent data from a half-sib breeding design and a selection experiment confirm the earlier ones with estimates for additive genetic variance ranging between 0.36 and 0.67 (Czesak & Fox 2003).

In contrast to sire effects, dam effects were highly significant throughout the present study, resulting in a relatively high dam component heritability (h^2 0.45 to 0.62). In contrast, Czesak and Fox (2003) found that egg size in the seed beetle *Stator limbatus* was only marginally affected by maternal effects variance (h^2 0.0 to 0.4). Our results suggest that either non-additive genetic variance exists or that egg size is rather strongly affected by the female's environment, phenotype or genotype. Assuming that dominance variance and epistatic interactions were zero, maternal effect variance is estimated to explain 10 to 14 % of the total phenotypic variance. The question of what pathways are involved in introducing such maternal effects on egg size remains unanswered, though we can largely exclude maternal environmental effects (see below). Thus, the most parsimonious explanation is that the maternal effects are due to female genotype. It is well known from other animals that even within single clones reared in a common environment, maternal identity effects can have a profound influence on offspring size (e.g. in *Daphnia;* Sakwinska 1994), and also the data from parent-offspring regressions suggest that maternal effects are important (see above). An alternative explanation would be the occurrence of non-additive genetic variance resulting in an overestimation of maternal effects variance.

Maternal environmental effects may evolve for cross-generational phenotypic plasticity, with mother's passing on their experience to the offspring to increase offspring fitness in predictable environments (Mousseau & Dingle 1991; Rossiter 1996; Fox & Mousseau 1998; Mousseau & Fox 1998). Although cross-generational maternal effects could potentially occur in *B. anynana* as this butterfly lives in a highly predictable environment with distinct seasons (Brakefield 1997), such a mechanism is unlikely to account for our results as all butterflies were reared for many generations under identical conditions.

Alternatively, maternal environmental effects could be mediated through differences in the quantity or quality of resources available to the mothers, and by maternal size or maternal age. None of those potential causes are likely to explain our results. First, all animals were reared under similar conditions within a single climate cell and were provided greenhouse-reared maize plants in ample supply. If differences in food quality or quantity among full-sib families (e.g. due to chance effects), potentially affecting the amount of resources available to reproduction, had been important, then similar maternal effects on pupal mass would be expected (for example, periods of starvation would cause a decrease of pupal mass for whole full-sib families). This, however, was not observed (see below). Second, maternal size is generally a poor predictor of egg size in *B. anynana* (see above; Fischer *et al.* 2002). Third, egg measurements were standardised for maternal age, as all egg measurements took place on days 4, 10 and 16 of adult life.

Heritability of pupal mass

Pupal mass was affected by both sire and dam effects. Both sources of variation influenced pupal mass to a similar extent, as is indicated by comparable variance components and heritability estimates. Additive genetic variance explained 25 % of the total phenotypic variance, well within the range found in other animals (e.g. 21 - 25 %, Kause *et al.* 1999; 25 %, Ueno 2003; for a review see Mousseau & Roff 1987). In contrast the mother's environment or phenotype had little influence on pupal mass.

In summary, our results demonstrate that in the butterfly, *B. anynana,* egg size responds in a plastic manner to oviposition temperature, that egg size is heritable and that there seems to be genetic variation in the plastic response to temperature.

These findings, together with earlier ones suggesting that temperature-mediated egg size plasticity in this particular species is adaptive (Fischer *et al.* 2003a,c), suggest that the conditions necessary for the evolution of phenotypic plasticity to occur are fulfilled. It is particularly noteworthy that the supposed existence of genetic variation in temperature reaction norms challenges the widely held notion that temperature-mediated plasticity (in egg and body size) might be purely a physiological constraint (e.g. Van der Have & De Jong 1996; Van Voorhies 1996). This notion mainly arises because of a lack of evidence for the adaptive significance of such plasticity (Partridge & French 1996; Ernsting & Isaaks 1997; Chown & Gaston 1999; Blanckenhorn 2000; Fox & Czesak 2000). A more comprehensive understanding of the adaptive significance of temperature-mediated plasticity as well as of genetic variation in temperature reaction norms is highly desirable.

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5.2. Within- and between-generation effects of temperature on life-history traits in a butterfly

Marc J. Steigenga[1]*, Klaus Fischer[1,2]

[1] Department of Animal Ecology I, Bayreuth University, D-95440 Bayreuth, Germany
[2] Zoological Institute, Greifswald University, D-17489 Greifswald, Germany

***Author for correspondence:**

Marc Steigenga
Department of Animal Ecology I
Bayreuth University
P.O. Box 101 251
D-95440 Bayreuth, Germany

Tel.: +49-921-553079
Fax: +49-921-552784
E-mail: marc.steigenga@uni-bayreuth.de

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Abstract

Non-genetic parental effects may largely affect offspring phenotype, and such plasticity is potentially adaptive. Despite its potential importance, little is known about cross-generational effects of temperature, at least partly because parental effects were frequently considered a troublesome nuisance, rather than a target of experimental studies. We here investigate effects of parental, developmental and acclimation temperature on life-history traits in the butterfly *Bicyclus anynana*. Higher developmental temperatures reduced development times and egg size, increased egg number, but did not affect pupal mass. Between-generation temperature effects influenced larval time, pupal time, larval growth rate and egg size analogously to developmental temperature, and additionally affected pupal mass but not egg number. Parental effects are important mediators of phenotypic plasticity in *Bicyclus anynana*, and partly yielded antagonistic effects on different components of fitness, which may constrain the evolution of cross-generational adaptive plasticity.

Introduction

The causes and consequences of phenotypic variation among individuals are of fundamental importance in evolutionary ecology, as it is this variation that provides the raw material for natural selection (Mousseau & Fox 1998). Two sources of phenotypic variation are readily recognized, namely the individual's genotype and the developmental environment (Schmalhausen 1949; Endler 1986; Wolf *et al*. 1998). However, in recent years it has become evident that the individual phenotype is often also affected by the environmental experience of other individuals (Mousseau & Dingle 1991; Mousseau & Fox 1998). In general inter-individual interactions occur most frequently between parents (primarily mothers) and their offspring (Mousseau & Fox 1998; Weigensberg *et al*. 1998; Wolf *et al*. 1998; Amarillo-Suárez & Fox 2006). Such non-genetic influences of parental phenotype or environment on progeny phenotype are of evolutionary importance not only because they influence short-term responses to selection (Kirkpatrick & Lande1989), but also because they are potentially adaptive (Mousseau & Dingle 1991; Rossiter 1996; Fox *et al*. 1997).

For example, if the parental environment is correlated with that of their offspring, then parents could enhance their own fitness by activating developmental programs that tune their offspring's phenotype for that environment (i.e. adaptive phenotypic

plasticity; Fox *et al*. 1997; Wolf *et al*. 1998; Gilchrist & Huey 2001). Environmental experience can be transmitted to offspring via cytoplasmic egg factors, e.g. yolk amount, egg composition, hormones or mRNA (Fox & Mousseau 1998; Mousseau & Fox 1998; Sakwinska 2004). One of the best studied examples for such cross-generational adaptive plasticity is the effect of the environmental conditions experienced by ovipositing insects on the diapause induction in the offspring (Mousseau & Dingle 1991; Huestis & Marahall 2006).

Although the potential importance of parental (mainly maternal) effects is well established now, there is still a relative lack of studies looking explicitly at such effects (Wolf *et al*. 1998; Lindholm *et al*. 2006), and little is known about cross-generational effects of temperature (Zamudio *et al*. 1995; Crill *et al*. 1996; Huey & Berrigan 1996; Gilchrist & Huey 2001; Stillwell & Fox 2005). This is at least partly because parental effects were frequently exclusively treated as a troublesome nuisance in quantitative genetic studies that need to be overcome by experimental design, rather than a target of experimental studies (Falconer & Mackay 1996; Mousseau & Fox 1998; Wolf *et al*. 1998; Andersen *et al*. 2005).

We have investigated within- and between-generation effects of temperature on development time, pupal mass and egg size in the tropical butterfly *Bicyclus anynana* (Butler 1879). We raised butterflies through two generations at high and low parental temperature, and afterwards for one generation at high and low developmental temperature. Additionally, ovipositing females were divided among two adult (i.e. acclimation) temperatures. We employed this design specifically to evaluate the relative importance of parental and developmental temperature (plus acclimation temperature for egg size and number; Crill *et al*. 1996). The effects of developmental and oviposition temperature as well as sex are already well established and therefore hardly novel (Fischer *et al*. 2003a,b, 2004, 2006a,b). However, their incorporation is nevertheless evidently useful in order to evaluate relative effect sizes and to explore potential interactions between parental and developmental / acclimation temperature.

We focused on temperature effects here as temperature is a key environmental factor for ectotherms (Hoffmann *et al*. 2003; Sinclair *et al*. 2003), and parental temperature has been found to have diverse phenotypic effects on offspring traits at

least in *Drosophila* (e.g. Zamudio *et al*. 1995; Crill *et al*. 1996; Huey and Berrigan 1996; Gilchrist and Huey 2001). Further, developmental and acclimation temperatures are known to affect the phenotype of many insects including *B. anynana* (Atkinson 1994; Atkinson *et al*. 2001; see above). Egg size, for instance, increases with decreasing temperature and there is evidence that this temperature-mediated egg size plasticity is adaptive in *B. anynana* (Fischer *et al*. 2003a,c). Finally, the heritability of egg size in *B. anynana* is fairly low with additive genetic variance explaining only 3 to 11 % of the total phenotypic variance (Steigenga *et al*. 2005). Maternal effects, in contrast, are much more pronounced, suggesting either substantial non-additive genetic variance or that egg size is rather strongly affected by maternal (or paternal) effects (Steigenga *et al*. 2005). This further prompted us to investigate the magnitude of parental environmental effects.

Materials and methods

2.1 Study organism

B. anynana is a tropical, fruit-feeding butterfly with a distribution ranging from Southern Africa to Ethiopia (Larsen 1991). The species exhibits striking phenotypic plasticity (two seasonal morphs), which is thought to function as an adaptation to alternative wet-dry seasonal environments and the associated changes in resting background and predation (Brakefield 1997; Lyytinen *et al*. 2004). Reproduction in this species is essentially confined to the warmer wet season when oviposition plants are abundantly available, and where 2-3 generations occur. Towards the end of the wet season there is a marked decrease in temperature, starting several weeks before larval food plants dry out. During the colder dry season reproduction ceases and butterflies do not mate before the first rains at the beginning of the next wet season (Windig 1994; Brakefield 1997). Morphs are gradually replaced during seasonal transitions. Thus, both phenotypes may occur simultaneously (Brakefield & Reitsma 1991).

A laboratory stock population of *B. anynana* was established at Bayreuth University, Germany, in 2003 from several hundred eggs derived from a well-established stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from over 80 gravid females caught at a single locality in Malawi. Several hundred adults are reared in each generation, maintaining

high levels of heterozygosity at neutral loci (Van't Hof *et al*. 2005). For this study butterflies from the Bayreuth stock population were used. Prior to setting up this experiment the stock population was reared for ca. six generations at 20°C.

2.2 General experimental design

To investigate effects of parental, developmental and acclimation (i.e. adult) temperature on several life-history traits in *Bicyclus anynana*, parental generation animals were raised at either 20 or 27°C (relative humidity 70 % and L:D 12:12 throughout). Each parental temperature group was subsequently divided among the same two temperatures (resulting in four treatment groups; two parental by two developmental temperatures). Resulting females were once again randomly divided among 20 and 27°C to test for effects of acclimation temperature on reproductive traits. The temperatures chosen are similar to the mean daily highs during the wet (27°C) and dry (20°C) season, respectively, in the field (Brakefield & Reitsma 1991; Brakefield & Mazzotta 1995). Thus, we did not include marginal temperatures, but ones the butterflies should be well adapted to.

2.3 Parental generation

To initiate this experiment, *B. anynana* eggs were collected from several hundred adults. Eggs were randomly divided among the high (27°C) and the low rearing temperature (20°C). To synchronize adult eclosion, two generations were subsequently reared at 27°C, but only one at 20°C (development at 20°C takes roughly twice as long compared to 27°C; Fischer *et al*. 2003a). Henceforth, the temperatures experienced during the last two generations before the synchronized eclosion of the offspring generation is referred to as 'parental temperature' (note that stock butterflies had been reared at 20°C previously, so no additional generation at 20°C was necessary). Larvae were kept in population cages and were fed on young maize plants. The resulting pupae were collected from the plants once daily and transferred to cylindrical hanging cages. Following adult eclosion, butterflies (> 150 per sex) were allowed to mate randomly within the two temperature groups at their respective rearing temperature.

2.4 Offspring generation

To initiate the next generation, females were allowed to lay eggs on small maize plants, which were replaced daily. These eggs, collected within 12 h light periods on several consecutive days, were once again randomly divided among 20 and 27°C for rearing (henceforth 'rearing temperature'). For rearing, eggs were transferred to elongated, sleeve-like gauze cages, separated by laying date. As egg hatching success and early larval survival may differ between temperatures (Fischer *et al* 2003a,c; Steigenga & Fischer in prep.), density was standardised to 40 larvae per cage after larvae had reached the third larval instar (to avoid detrimental handling effects). Larval mortality is negligible from this stage onwards. Per temperature treatment (i.e. 20-20, 20-27, 27-20, 27-27 for 'parental' and offspring 'rearing temperature'), 10 replicate cages were kept. The cages' positions within climate chambers were randomized daily. Throughout, only two different climate cabinets (i.e. one per temperature) with 20 and 27°C, respectively, were used.

The resulting pupae were collected once daily, sexed, and weighed to the nearest 0.01 mg on the day following pupation. Afterwards, they were placed individually in translucent, numbered plastic pots (125 ml) until adult eclosion, after which female butterflies were marked individually. They were kept separate from males for two days, after which they were set up for mating for two days with random males from their temperature group. Then, females were placed individually in translucent plastic containers (1L, covered with gauze), containing a fresh cutting of maize to oviposit on. Two days later (i.e. on day six of adult life), all eggs were removed, counted and subsequently measured (see below). While these first eggs were collected at the females' respective rearing temperature, females were thereafter a last time randomly divided among 20 and 27°C to induce a plastic response in egg size (henceforth 'oviposition temperature'). This resulted in a total of eight treatment groups: 20-20-20, 20-20-27, 20-27-20, 20-27-27, 27-20-20, 27-20-27, 27-27-20, 27-27-27 (for 'parental', 'rearing' and 'oviposition temperature'). Eggs were once again collected and measured after an acclimation period of 10 days (i.e. on day 18 of adult life). In *B. anynana* a clear plastic response in egg size to temperature can be expected to occur within this period (Fischer *et al.* 2003a,b,c; Steigenga *et al*. 2005). During acclimation females had continuous access to oviposition plants. Throughout all experiments, butterflies had access to moist banana for adult feeding.

For all individuals we measured larval time (from egg laying to pupation, thus including egg development time), pupal time (from pupation to adult eclosion) and pupal mass. Growth rates were calculated as [LN (pupal mass) / larval time] x 100 (cf. Nylin 1992; Fischer & Fiedler 2000). For females, additionally egg sizes and numbers for days 5-6 and 17-18 of adult life were scored.

2.5 Egg measurements

As the eggs of *B. anynana* are nearly perfect spheres, egg size was measured as cross-sectional projections (mm^2) using a digital camera (Leica DC300, Leica Microsystems, Wetzlar, Germany) connected to a binocular microscope. The resulting images were analysed using Scion Image public software (Scion Corporation 2000). Tight correlations between egg area (applying image analysis) and egg mass as well as hatchling size confirm that this method provides a highly reliable measurement of egg size in *B. anynana* (Fischer *et al*. 2002). To calculate egg size for individual females, the mean of ca. 10 eggs per female and collection period was used.

2.6 Statistical analysis

Data were analysed using analyses of (co-)variance (AN(C)OVAs) with parental temperature, rearing temperature as well as oviposition temperature or sex as fixed factors. Replicate cage (random factor, nested within rearing temperature) and pupal mass (covariate) were added as appropriate. Using replicate cage means as the level of replication did not yield qualitatively different results, except that the interaction between rearing temperature and sex for pupal mass became non-significant, and that pupal mass differed significantly between rearing temperatures. If necessary, data were transformed prior to analyses to meet ANOVA requirements. Significant differences between individual groups were identified using Tukey's HSD. All statistical tests were performed using Statistica 6.1. Throughout all means are given ± 1 SE.

Results

3.1 Developmental traits

Both parental and rearing temperature affected (or tended to affect in case of rearing temperature and pupal mass) all developmental traits measured (Table 1A and Figure 1). Generally, variation induced by the respective rearing temperature was much more pronounced than that induced by parental temperature, except for pupal mass. Here, pupal mass was consistently higher when parents were raised at 20 compared to 27°C (187.9 ± 1.7 vs. 177.9 ± 1.7 mg), regardless of rearing temperature (see Table 1A for statistics). Likewise, larval (36.3 ± 0.2 vs. 32.6 ± 0.2 days) and pupal (10.6 ± 0.1 vs. 9.7 ± 0.1 days) development time were longer and concomitantly growth rate (16.8 ± 0.1 vs. 18.3 ± 0.1 %/day) lower when parents were reared at the lower temperature. Regarding rearing temperature, larval (47.6 ± 0.3 vs. 22.1 ± 0.3 days) and pupal (14.4 ± 0.14 vs. 6.1 ± 0.13 days) development time were much longer and growth rate (11.0 ± 0.1 vs. 23.8 ± 0.1 %/day) was lower at 20 compared to 27°C. Pupal mass tended to be higher at the higher rearing temperature (185.7 ± 1.7 vs. 180.4 ± 1.8 mg).

There were significant interactions between parental and rearing temperature for larval time, pupal time and growth rate, reflecting, in absolute terms, a stronger effect of parental temperature for larval and pupal time when animals were raised at a rearing temperature of 20 compared to 27°C, but the opposite for growth rate (Figure 1). In relative terms, however, changes were very similar across parental temperatures except for pupal time. For all traits except pupal time there was additionally significant variation across replicate cages.

Further, all developmental traits differed significantly between sexes (Table 1A and Figure 1). Overall, females had longer larval times (35.8 ± 0.2 vs. 33.3 ± 0.2 days), marginally shorter pupal times (10.1 ± 0.1 vs. 10.2 ± 0.1 days), higher pupal masses (192.1 ± 1.7 vs. 174.0 ± 1.7 mg) and lower growth rates (17.0 ± 0.1 vs. 18.1 ± 0.1 %/day) than males. Significant interactions between rearing temperature and sex were present for pupal time, pupal mass and growth rate, suggesting that sex differences tended to be more pronounced at 27 than at 20°C for pupal mass and growth rate, but the reverse for pupal time (Figure 1).

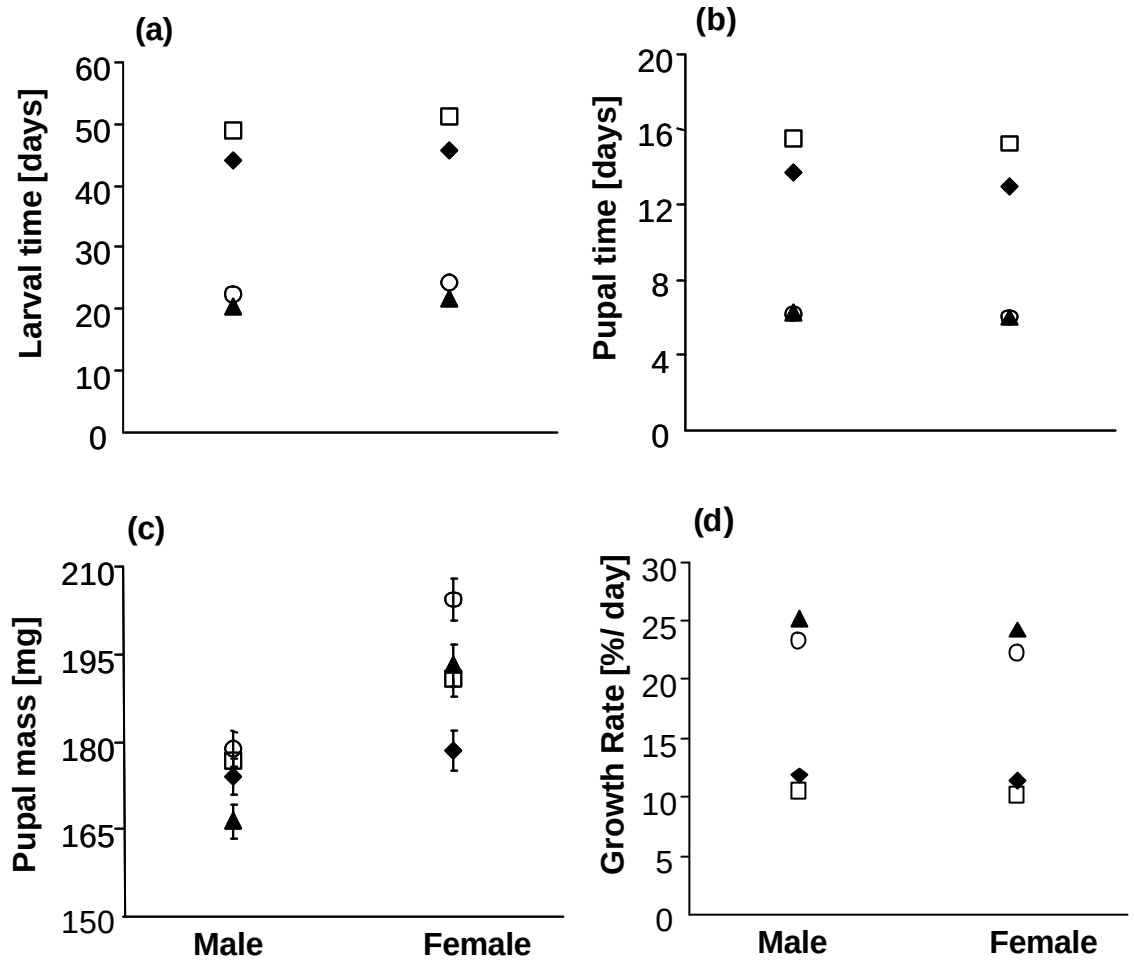

Figure 1. *Effects of parental and rearing temperature as well as sex on larval time (a), pupal time (b), pupal mass (c) and growth rate (d) in Bicyclus anynana. Squares: 20-20 (parental temperature 20°C/rearing temperature 20°C); diamonds: 27-20; circles: 20-27, triangles: 27-27. Open symbols: parental temperature: 20°C; filled symbols: parental temperature: 27°C. Standard error bars for data points are smaller than the symbols in some cases. Sample sizes range between 77 and 96 per group.*

2.2 Egg size and number I

Size and number of the eggs laid first were not (*per se*) affected by parental temperature, oviposition temperature or pupal mass, but differed significantly among rearing temperatures (Table 1B and Figure 2). Females reared (and maintained until day six of adult life) at the lower temperature laid significantly larger (0.723 ± 0.005 vs. 0.671 ± 0.004 mm^2) but fewer (11.0 ± 1.7 vs. 26.6 ± 1.4) eggs compared to females reared at the higher temperature (Figure 2). A significant interaction between

parental and rearing temperature for egg size indicates that the differences induced by rearing temperature were more pronounced when parents were raised at 20 rather than at 27°C (Figure 2a). The significant interaction between parental and oviposition temperature is considered a chance effect of allocation to treatment groups, as egg size was measured prior to division among oviposition temperatures.

2.3 Egg size and number II

As above, egg size and number of the eggs laid on days 17-18 of adult life were not (*per se*) affected by parental temperature or pupal mass (Table 1B and Figure 2). However, rearing and oviposition temperature (tended to cause or) caused significant variation. Females reared at 20°C laid larger (0.701 ± 0.005 vs. 0.669 ± 0.006 mm^2) and more (21.3 ± 1.3 vs. 17.2 ± 1.4) eggs than those reared at 27°C. Regarding oviposition temperature, egg size was larger (0.698 ± 0.005 vs. 0.675 ± 0.005 mm^2; except for the females reared at 27°C when their parents were raised at 20°C) and egg number tended to be lower (17.3 ± 1.3 vs. 21.0 ± 1.3) at 20°C compared to 27°C (Figure 2). A significant interaction between parental and rearing temperature for egg size once again suggests that differences among rearing temperature groups were more pronounced when parental generation butterflies were raised at the lower temperature (Figure 2a). The significant three-way interaction for the same trait reflects the substantial variation in the plastic response to oviposition temperature among thermal groups, with eggs being substantially larger, somewhat larger or even smaller at 20 compared to 27°C (Figure 2a).

Next pages: **Table 1.** *Results of AN(C)OVAs for the effects of parental temperature, rearing temperature, replicate cage (random factor, nested within rearing temperature) and sex on larval time, pupal time, pupal mass and growth rate (A, next page) as well as for the effects of parental temperature, rearing temperature (including the first 6 days of adult life during which first eggs were laid), oviposition temperature (from day 6 of adult life onwards) and pupal mass (covariate) on egg sizes and numbers (B, page 66) in Bicyclus anynana. Significant P-values are given in bold. 'Egg size/number I' refers to the eggs produced while still being at the females' rearing temperature, 'egg size/number II' to those produced at the oviposition temperature. T: temperature.*

Table 1A.

Trait	Source	df	MS	*F*	*P*
Larval time	Parental T	1,658	1940.1	249.5	**< 0.0001**
	Rearing T	1,20	102328.0	3229.9	**< 0.0001**
	Replicate [Rearing T]	20,658	32.5	4.9	**< 0.0001**
	Sex	1,658	531.8	68.4	**< 0.0001**
	Parental T x Rearing T	1,658	304.7	39.2	**< 0.0001**
	Parental T x Sex	1,658	32.3	3.0	0.0839
	Rearing T x Sex	1,658	7.4	1.0	0.3287
	3-Way Interaction	1,658	8.0	1.0	0.3119
Pupal time	Parental T	1,658	148.8	249.5	**< 0.0001**
	Rearing T	1,20	10751.8	3229.9	**< 0.0001**
	Replicate [Rearing T]	20,658	6.1	4.9	**< 0.0001**
	Sex	1,658	19.9	68.4	**< 0.0001**
	Parental T x Rearing T	1,658	167.2	39.2	**< 0.0001**
	Parental T x Sex	1,658	1.5	3.0	0.2165
	Rearing T x Sex	1,658	4.2	1.0	**0.0370**
	3-Way Interaction	1,658	0.6	1.0	0.4304
Pupal mass	Parental T	1,658	17264.2	16.3	**< 0.0001**
	Rearing T	1,20	3781.5	4.0	0.0598
	Replicate [Rearing T]	20,658	958.8	1.1	0.3774
	Sex	1,658	50200.8	56.0	**< 0.0001**
	Parental T x Rearing T	1,658	35.4	< 0.1	0.8426
	Parental T x Sex	1,658	775.4	0.9	0.3527
	Rearing T x Sex	1,658	12731.3	14.2	**< 0.0001**
	3-Way Interaction	1,658	956.3	1.1	0.3020
Growth rate	Parental T	1,658	0.0330	127.4	**< 0.0001**
	Rearing T	1,20	2.6122	4211.6	**< 0.0001**
	Replicate [Rearing T]	20,658	0.0006	2.5	**0.0005**
	Sex	1,658	0.0087	33.8	**< 0.0001**
	Parental T x Rearing T	1,658	0.0029	11.4	**< 0.0008**
	Parental T x Sex	1,658	< 0.0001	0.1	0.7442
	Rearing T x Sex	1,658	0.0020	7.9	**0.0052**
	3-Way Interaction	1,658	< 0.0001	< 0.1	0.8830

Table 1B.

Trait	Source	Df	MS	*F*	*P*
Egg size I	Parental T	1,217	0.0002	< 0.1	0.7703
	Rearing T	1,217	0.1287	54.5	**< 0.0001**
	Ovipos. T	1,217	0.0002	< 0.1	0.7844
	Parental T x Rearing T	1,217	0.0251	10.6	**0.0013**
	Parental T x Ovipos. T	1,217	0.0130	5.5	**0.0197**
	Rearing T x Ovipos. T	1,217	0.0045	1.9	0.1694
	3-Way Interaction	1,217	0.0003	0.1	0.7131
	Pupal Mass	1,217	0.0030	1.3	0.2618
Egg size II	Parental T	1,207	< 0.0001	< 0.1	0.8972
	Rearing T	1,207	0.0393	13.9	**0.0003**
	Ovipos. T	1,207	0.0232	8.2	**0.0047**
	Parental T x Rearing T	1,207	0.0223	7.9	**0.0055**
	Parental T x Ovipos. T	1,207	0.0037	1.3	0.2560
	Rearing T x Ovipos. T	1,207	0.0007	0.3	0.6146
	3-Way Interaction	1,207	0.0238	8.4	**0.0042**
	Pupal Mass	1,207	0.0020	0.7	0.4002
Egg Nr I	Parental T	1,217	187.8	0.7	0.3938
	Rearing T	1,217	12688.9	49.3	**< 0.0001**
	Ovipos. T	1,217	202.2	0.8	0.3763
	Parental T x Rearing T	1,217	10.1	< 0.1	0.8433
	Parental T x Ovipos. T	1,217	54.7	0.2	0.6450
	Rearing T x Ovipos. T	1,217	61.7	0.2	0.6242
	3-Way Interaction	1,217	15.2	0.1	0.8080
	Pupal Mass	1,217	24.6	0.1	0.7575
Egg Nr II	Parental T	1,207	43.1	0.2	0.6318
	Rearing T	1,207	900.6	4.8	**0.0294**
	Ovipos. T	1,207	652.6	3.5	0.0632
	Parental T x Rearing T	1,207	90.1	0.5	0.4886
	Parental T x Ovipos. T	1,207	384.2	2.1	0.1534
	Rearing T x Ovipos. T	1,207	895.5	4.8	**0.0298**
	3-Way Interaction	1,207	344.0	1.8	0.1766
	Pupal Mass	1,207	74.4	0.4	0.5289

For egg number only the interaction between rearing and oviposition temperature was significant, demonstrating that oviposition temperature did not affect egg number when first eggs were laid at 27°C, while more eggs were laid at the higher oviposition temperature when females had laid their first eggs at 20°C (Figure 2b).

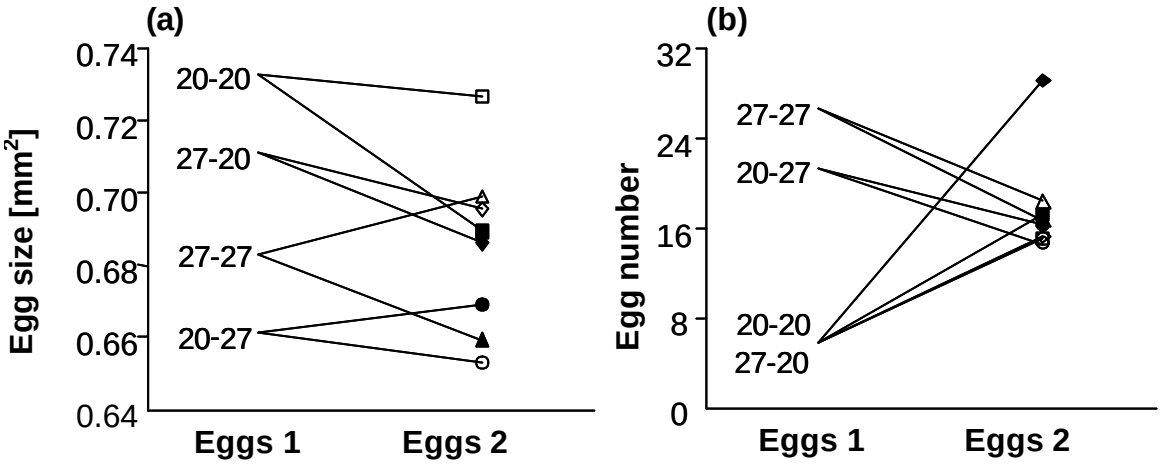

Figure 2. *Effects of parental, rearing and oviposition temperature on mean egg size (a) and number (b) in Bicyclus anynana. 20-20: parental temperature 20°C/rearing temperature 20°C; 20-27: parental temperature 20°C/rearing temperature 27°C and so on. Note that the 'rearing temperature' includes the first 6 days of adult life during which first eggs (Eggs 1) were laid, while later eggs (Eggs 2) were laid at the respective oviposition temperature where butterflies were kept from day 6 of adult life onwards. Open symbols: oviposition temperature 20°C; filled symbols: oviposition temperature 27°C. For better visibility no standard errors are given. Sample sizes range between 18 and 40 per group.*

Discussion

4.1 Between-generation effects

In this study we found substantial effects of developmental and acclimation temperature on life-history traits (i.e. within-generation effects; see below), but also of the temperature experienced in the parental generation (i.e. between-generation or carry-over effects). While within-generation effects of temperature have received considerable attention in recent decades (e.g. Atkinson 1994; Partridge & French 1996; Chown & Gaston 1999; Atkinson *et al*. 2001), comparatively little is known about the frequency of occurrence and magnitude of between-generation effects

(Crill *et al*. 1996; Huey & Berrigan 1996; Gilchrist & Huey 2001; Stillwell & Fox 2005). Such effects substantially affected larval time, pupal time, pupal mass, larval growth rate, and even egg size through its interactions with rearing temperature in the present study. Parental carry-over effects are usually most pronounced early in life and diminish when offspring matures (e.g. Mousseau & Dingle 1991; Crill *et al.* 1996; Wolf *et al*. 1998; McAdam *et al*. 2002; Sakwinska 2004; Lindholm *et al*. 2006).

This notion agrees with our results as larval development time and growth rate were strongly affected by parental temperature in *B. anynana* (cf. Roff & Sokolovska 2004). Additionally, pupal time and mass were affected across generations, which seems to be quite rare in animals (but Crill *et al*. 1996; Sakwinska 2004), though environmentally-induced phenotypic variation across multiple generations has been detected in many studies with plants (Bernardo 1996a).

Regarding effect directions, animals whose parents had been raised at the lower temperature had longer larval and pupal times, a concomitantly reduced larval growth rate, and an increased pupal mass compared to those whose parents had been raised at the higher temperature. These patterns are in broad agreement with those previously reported for *Drosophila* (Crill *et al*. 1996; Gilchrist & Huey 2001), and closely resemble the effects typically induced by differences in developmental temperature. Low developmental temperatures are well known to constrain development time and to affect body size according to the 'temperature-size rule' (Honek & Kocourek 1990; Atkinson 1994; Partridge & French 1996; Chown & Gaston 1999; Atkinson *et al*. 2001; Fischer *et al*. 2003a). Thus, parental and prevailing developmental conditions may result in very similar patterns of variation in life-history traits, as has been previously documented for the within-generation effects of rearing (a parental effect here) and oviposition temperature on egg size and hatching success (Fischer *et al*. 2003b, 2004, 2006b; but Stillwell & Fox 2005).

Hitherto, nothing is known about the mechanistic basis of cross-generational effects in *B. anynana*, as is the case for most other arthropods except *Drosophila* (Rushlow *et al*. 1987; Girton & Leon 1994). The factors triggering such effects need to be transferred to the offspring via the egg stage. While it seems rather unlikely that egg size *per se* is of crucial importance, variation in egg content might at least in part be

involved. Environmental experience might be transmitted to offspring via cytoplasmic egg factors, e.g. yolk amount, egg composition, hormones or mRNA (Bernardo 1996b; Fox & Mousseau 1998; Mousseau & Fox 1998; Fox *et al*. 2004; Sakwinska 2004; Amarillo-Suárez & Fox 2006). However, we do not yet have data on temperature-mediated variation in egg composition and its specific effects in *B. anynana* (work in progress). Generally, the importance of variation in egg composition rather than egg size has received comparably little attention (Royle *et al*. 1999; Giron & Casas 2003; Fischer *et al*. 2006a; Karl *et al*. in press).

4.2 Within-generation effects

As expected, development times were much longer and larval growth rates much lower when animals were reared at a lower temperature (see above; Honek & Kocourek 1990; Fischer *et al*. 2003a). Contrary to our expectations based on the temperature-size rule (e.g. Atkinson 1994), however, pupal mass was not significantly affected by rearing temperature and even tended to increase at the higher temperature. This may suggest that, regarding pupal mass as opposed to development times, parental and developmental temperature did not interact in a synergistic, but rather antagonistic manner (see above; see also Crill *et al*. 1996). The significant interactions between parental and rearing temperature for larval time, pupal time and growth rate indicate that rearing temperature effects depend at least to some extent on the environment experienced by the parents, but with very little effect on grand patterns.

Regarding egg size, females reared at the lower temperature produced larger eggs compared to those being reared at the higher temperature (Avelar 1993; Ernsting & Isaaks 1997; Blanckenhorn 2000; Fischer *et al*. 2003b, 2004, 2006b; Steigenga *et al*. 2005). This developmental (and early adult) temperature effect persisted through to the second measurement, ten days after the division among oviposition temperatures (see also Fischer *et al*. 2003b). Then, egg size was additionally affected by oviposition temperature (Fischer *et al*. 2003a,b,c, 2004, 2006b). Although the effects of the rearing temperature were still substantial at this point in time (see also e.g. Fox & Savalli 1998; Roff & Sokolovska 2004 for long-lasting maternal effects), such developmental effects can at least sometimes, given enough time, be largely reversed by acclimation in the adult stage (Fischer *et al.* 2003b). An interaction

between parental and developmental temperature for egg size indicates some influence of the temperature environment experienced in the previous generations, but again with very little impact on grand patterns.

The fact that the females laying larger eggs at the time of first egg measurements also laid fewer ones does not suggest a resource-allocation trade-off (note that variation in egg size is relatively small compared to variation in egg number; see also Fischer *et al*. 2003a), but rather that a temperature of 20°C is suboptimal for this tropical butterfly and substantially constrains activity levels and presumably also egg maturation (Ellers & Boggs 2004; Steigenga & Fischer in prep.). At the time of second egg measurements, egg numbers once again tended to be lower at 20 than at 27°C. However, this effect was entirely restricted to females having laid their first eggs at the lower temperature (as indicated by a significant rearing by oviposition temperature interaction). This effect results from the fact that the females having spent their previous time at 20°C still have a large fraction of their eggs available for deposition, while those females kept previously at 27°C are either 'constrained' by the prevailing egg-laying temperature (those ovipositing at 20°C) or have already laid a large fraction of their eggs before.

Egg size is generally believed to be closely related to fitness (Fox & Czesak 2000) and recent studies of *B. anynana* showed that females reared at a lower temperature laid larger eggs with increased fitness than those reared at higher temperatures (Fischer *et al*. 2003a,b,c, 2004, 2006b). As the environment the mother experiences is a good predictor for the environment the offspring will experience in *Bicyclus anynana* (Windig 1994; Brakefield 1997), mothers could enhance their own fitness by tuning their offspring's reproductive physiology (i.e. adaptive phenotypic plasticity; Fox *et al*. 1997; Wolf *et al*. 1998; Gilchrist & Huey 2001), thereby optimising survival and fitness in this butterfly.

4.3 Sex-specific differences

The sex-specific differences in life-history traits found were as expected and confirm existing data (e.g. Fischer *et al*. 2003a, 2006b). Earlier male emergence due to short development times is frequently found in insects and is commonly attributed to protandry selection (Wiklund & Fagerström 1977; Fagerström & Wiklund 1982;

Wiklund *et al*. 1991; Fischer & Fiedler 2000; Zijlstra *et al*. 2002). Such a developmental advantage is typically achieved by higher male growth rates as is the case here (Wiklund *et al*. 1991; Fischer & Fiedler 2000, 2001). In this context the slightly longer pupal development in males appears to be enigmatic, though this pattern has been repeatedly documented in *B. anynana*, but has very little effect on the overall sex difference in development time (Fischer *et al*. 2003a, 2006a). A larger body size in female insects, finally, is considered to be the result of fecundity selection (Honek 1993; Roff 2002; for *B. anynana* see Fischer *et al*. 2006a).

Apart from these well-known patterns it is noteworthy that the effects of rearing temperature differed at least partly between the sexes. Rearing temperature by sex interactions were present for pupal mass (not if cage means were used as the level of replication), pupal time and larval growth rates. Sex-specific temperature reaction norms were also found in an earlier study using *B. anynana* (Fischer *et al*. 2006b; see also Fischer & Fiedler 2000, 2001; Fairbairn 2005; Teder &Tammaru 2005). Also in the earlier study, female body size responded more strongly to different rearing temperatures than male body size. A potential mechanism behind such sexual differences in phenotypic plasticity might be differential canalisation of body size and reproductive traits (Fairbairn 2005; Teder & Tammaru 2005).

The interactions for pupal time and larval growth rates need to be interpreted with caution. Typically, such interactions reflect differences in absolute terms, while relative differences (e.g. expressing male development times relative to female development times) are very similar, which is also the case here.

Conclusions

Our data show that life-history traits can be substantially affected by both, within- and between-generation temperature effects. Such parental environmental effects may evolve for cross-generational adaptive plasticity, with parents 'preparing' their offspring for the environmental conditions they are likely to encounter (Mousseau & Dingle 1991; Rossiter 1996; Mousseau & Fox 1998). As the amount and quality of resources invested into eggs by female insects may profoundly influence offspring growth and survival, insect eggs are considered important mediators of maternal effects (Fox 1993, 1994; Bernardo 1996b; Azevedo *et al*.1997; Fox & Mousseau

1998; Fox & Czesak 2000). The adaptive significance of cross-generational temperature effects, however, is hitherto largely unresolved (Gilchrist & Huey 2001). Our study was not designed to explicitly test for any associated fitness consequences, though some tentative conclusions seem possible.

Regarding development times and assuming that a quick development is at a selective premium (e.g. due to reduced exposure time to predators and parasitoids or by enabling rapid reproduction; Stearns 1992; Partridge & French 1996; Roff 2002), we found no general advantage to offspring when parents had been raised in the same environment. Thus, there was no support for the 'adaptive cross-generational hypothesis' (Gilchrist & Huey 2001). Rather, development times were shorter when parents had been raised at the higher temperature, supporting the 'hotter parents are better hypothesis' (Gilchrist & Huey 2001). For pupal mass, in contrast, there is clear support for the 'colder parents are better hypothesis' (Gilchrist & Huey 2001), as these produced substantially larger offspring compared to parents that had been reared at the higher temperature. Larger body size, in turn, is frequently considered beneficial due to its positive effects on fecundity, metabolic rates, competitive abilities etc. (Schmidt-Nielsen 1984; Blanckenhorn 2000; Fischer *et al*. 2006a). Thus, the parental environment seems to have antagonistic effects on different components of fitness, and consequently no firm conclusions about any 'optimal' parental temperature seem possible. Further note that our design does not allow us to test for other competing hypothesis, namely the 'intermediate temperature is best' hypothesis (Gilchrist & Huey 2001).

Regarding egg size, there were clear effects of developmental temperature (which is a parental effect on egg size; see above). These were in line with previous findings and are likely to be adaptive in *B. anynana*. Recent studies showed that females reared at a lower temperature laid larger eggs with increased fitness than those reared at higher temperatures (Fischer *et al*. 2003a,b,c; 2004; 2006b). However, trans-generational effects, though present through interactions, had only marginal effects on egg size.

Summary

Our study shows that not only developmental and acclimation temperature affect life-history traits, but also the parental thermal environment. Such effects may not only affect early development, but may also persist through to the adult stage. Generally, maternal effects are widespread, but have been seldom documented for differences in temperature environments. Our study shows that the thermal environment experienced in different generations and at different time points may interact in a complex manner, making any predictions about effect directions evidently difficult. Further, carry-over effects may yield antagonistic effects on different components of fitness, which may constrain the evolution of cross-generational adaptive plasticity.

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6

The mechanistic background of temperature mediated reproductive plasticity

6.1 Effects of the juvenile hormone mimic pyriproxyfen on female reproduction and longevity in the butterfly *Bicyclus anynana*

Marc J. Steigenga[1]*, Klaus H. Hoffmann[1] and KlausFischer[1]

[1] *Department of Animal Ecology I, Bayreuth University, D-95440 Bayreuth, Germany*

***Author for correspondence:**

Marc Steigenga
Department of Animal Ecology I
Bayreuth University
P.O. Box 101 251
D-95440 Bayreuth, Germany

Tel.: +49-921-553079
Fax: +49-921-552784
E-mail: marc.steigenga@uni-bayreuth.de

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Abstract

Female *Bicyclus anynana* butterflies given pyriproxyfen, a mimic of juvenile hormone, exhibited increased egg-laying rates and early fecundity, but reduced longevity compared to control animals. Thus, pyriproxyfen application yielded antagonistic effects on different components of fitness, possibly demonstrating a juvenile hormone-mediated trade-off between present and future reproduction. Lifetime fecundity and egg size, however, showed no consistent response to pyriproxyfen, with lifetime fecundity being increased or decreased and egg size being reduced in one out of four experiments only. Females were most sensitive to pyriproxyfen around the onset of oviposition, coinciding with naturally increasing juvenile hormone titres in other Lepidoptera. Amounts between 1 and 10 µg pyriproxyfen were found to be effective, with, however, pronounced differences among experiments. This is attributed to differences in assay conditions. High pyriproxyfen concentrations (100 µg) as well as repeated applications of smaller amounts did not affect reproductive traits, but tended to reduce longevity.

Introduction

In insects, hormones are the main regulators of life-history components like metamorphosis, behaviour, caste determination, diapause, polymorphisms and reproduction (Edwards *et al*. 1995; Gäde *et al*. 1997; Nijhout 1998; Flatt *et al.* 2005). The principle hormones influencing these components are the juvenile hormones (JHs henceforth) and the ecdysteroids. As the biosynthesis of JH depends on environmental conditions such as temperature and photoperiod, they are frequently involved in mediating life-history plasticity (e.g. Dingle & Winchell 1997; Zera *et al*. 1998; Emlen & Nijhout 1999). Differences in JH titres in turn can affect reproductive output (e.g. Cusson *et al*. 1990; Trumbo & Robinson 2004).

In our study organism *Bicyclus anynana* (Butler 1879) (Lepidoptera: Nymphalidae), a tropical butterfly, environmentally-induced differences in reproductive strategies exist. Recent studies showed that females kept at a lower oviposition temperature laid larger but fewer eggs than those kept at higher temperatures (Fischer *et al*. 2003a,b). The available evidence suggests that it is profitable to produce fewer but larger offspring (with increased fitness) at a lower temperature, and more and smaller offspring at a higher temperature where offspring survival is generally high in this

butterfly (Fischer *et al*. 2003a,b). The hormonal background of these differences is unknown, but JHs are suspected to be involved in triggering this temperature-mediated plasticity.

Generally, JH is the most important hormone involved in insect reproduction, where it has basically two functions. Its primary function is to initiate vitellogenin synthesis, and its secondary to regulate the uptake of yolk by the ovary (Hoffmann 1995). Based on the hormones being used for initiation of vitellogenesis and the timing of egg production, the Lepidoptera can be divided into four groups (Ramaswamy *et al*. 1997). One of these consists of Lepidoptera that are completely independent of metamorphic events for vitellogenesis and choriogenesis, which are initiated and completed after adult emergence. In this group, including several butterfly species, JH is the only hormone necessary for synthesis of vitellogenin in the fat body, inducing patency of ovarioles, uptake of vitellogenin and choriogenesis (Ramaswamy *et al*. 1997). The life history of *B. anynana* closely matches all criteria of this particular group (cf. Ramaswamy *et al*. 1997), as this species is polyandrous (Brakefield *et al*. 2001) and because adult-derived carbohydrates are needed for the onset of oviposition (Fischer *et al*. 2004; Bauerfeind & Fischer 2005).

Given the anticipated importance of JH for egg development in this butterfly and the widespread role of JH in mediating life-history plasticity (see above), we here examine the effects of a JH mimic (pyriproxyfen) on reproductive output in *B. anynana*, also in search of a potential candidate for triggering the temperature-related plastic responses outlined above. In particular, we address the following issues: (1) Are egg size and fecundity affected by pyriproxyfen? (2) If yes, when is the sensitive period? (3) Are the (presumed) effects dose-dependent? Note that, while the effects of JH on ovarian and egg development are fairly well understood (for Lepidoptera e.g. Pan & Wyatt 1971, 1976; Herman & Bennett 1975; Satyanarayana *et al*. 1991, 1992), data on its effects on reproductive output are scarce (Rankin *et al*. 1997; Trumbo & Robinson 2004), especially in Lepidoptera (e.g. Ramaswamy *et al*. 1997; Webb *et al*. 1999). As we, along with egg size and fecundity, also scored longevity across treatment groups, we will additionally explore the presumed trade-off between present and future reproduction (e.g. Herman & Tatar 2001; Tatar & Yin 2001; Zera & Harshman 2001).

Materials and methods

Study organism

B. anynana is a tropical, fruit-feeding butterfly with a distribution ranging from Southern Africa to Ethiopia (Larsen 1991). The species exhibits striking phenotypic plasticity (two seasonal morphs), which is thought to function as an adaptation to alternative wet-dry seasonal environments and the associated changes in resting background and predation (Brakefield 1997; Lyytinen *et al*. 2004). A laboratory stock population was established at Bayreuth University, Germany, in 2003 from several hundred individuals derived from a well-established stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from about 80 gravid females caught at a single locality in Malawi. Several hundred adults are reared in each generation, maintaining high levels of heterozygosity at neutral loci (Van't Hof *et al*. 2005). For this study butterflies from the Bayreuth stock population were used.

Butterfly rearing

Throughout, all butterflies were reared and maintained in a climate room at 27°C, high relative humidity (70 %), and a photoperiod of L12:D12. Larvae were fed on young maize plants and kept in population cages (50 x 50 x 80 cm). The resulting pupae were collected from the plants and transferred to cylindrical hanging cages. Following adult eclosion (day 0), males and females were kept separate for two days. Afterwards females were set up for mating for two days with an equal number of random males, following which they were placed individually in labelled 1 L plastic containers with a fresh maize leaf for oviposition. Eggs were collected and measured (see below) every other day (starting on day 6 of adult life) until the death of the females. Throughout all experiments, butterflies had access to moist banana for adult feeding.

Experimental design

To investigate the effects of pyriproxyfen (4-phenoxyphenyl (*RS*)-2-(2-pyridyloxy) propyl ether; > 99.0 % purity; purchased from Dr. Ehrenstorfer Ltd., D-86199 Augsburg, Germany) on female *B. anyana* reproduction we performed four different experiments as outlined below. Pyriproxyfen was chosen because of its higher

activity and stability as compared to JHs. Throughout pyriproxyfen was dissolved in 3 µl hexane or acetone, and was applied topically on the females' abdomen using a blunt 10-µl syringe. Solutions were remade every other day.

Experiment 1: Time series

Here, *B. anynana* females were treated with 10 µg pyriproxyfen dissolved in hexane, as this concentration is known to work well in other insects (Edwards *et al*. 1993; Rankin *et al*. 1997; Zeng *et al*. 1997; Zera *et al*. 1998). To characterize the sensitive period, pyriproxyfen was applied on either the eclosion day (day 0 of adult life), before mating (day 2), before the onset of oviposition (day 4) or during oviposition (day 6). Controls received 3 µl pure hexane (i.e. without pyriproxyfen) on the eclosion day.

Experiment 2: Concentration series

To investigate the dose-dependence of presumed changes in reproductive traits, females were treated with different concentrations of pyriproxyfen (1, 10 or 100 µg in 3 µl hexane), all of which were applied on day 4 of adult life (i.e. just before the onset of oviposition; butterflies showed the highest sensitivity to pyriproxyfen at this time point in *experiment 1*). Controls received 3 µl pure hexane the same day.

Experiment 3: Repeated pyriproxyfen applications

As JH mimics are known to degrade quite rapidly especially when applied topically (Edwards *et al*. 1993; Gäde *et al*. 1997; Engelmann & Mala 2000), and because continuously high hormone levels may be needed to demonstrate clear effects on reproduction, pyriproxyfen (1 or 5 µg in 3 µl acetone; hexane was considered too poisonous for repeated applications) was applied on seven consecutive days after eclosion. Higher concentrations of pyriproxyfen were not included because the above experiment showed that such concentrations are deleterious even when applied only once. Controls were treated with 3 µl acetone the same days.

Experiment 4: 'A closer look'

As effects were found to be quite subtle in previous experiments, a last experiment using the time periods and concentrations proven to be most effective, combined with large sample sizes to increase statistical power, was carried out. Females received 5

µg pyriproxyfen in 3 µl acetone either during mating (day 3 of adult life), before the onset of oviposition (day 4) or on the first day of egg laying (day 5). To control for possible time dependent detrimental effects of the solvent, three control groups that received 3 µl pure acetone on days 3, 4 or 5 were established, resulting in a highly controlled experimental design. Here, also reproductive investment was calculated as the product of lifetime fecundity and mean egg size (averaged over the whole oviposition period). Data were transformed into mg using a formula given by Fischer *et al*. (2002). Comparable estimates were not needed in the experiments before as egg size did not differ across treatments.

Egg measurements

As the eggs of *B. anynana* are nearly perfect spheres egg size was measured as cross-sectional area (mm^2) using a digital camera (Leica DC300, Leica Microsystems, Wetzlar, Germany) connected to a stereo microscope. The resulting images were analysed using Scion Image public software (Scion Corporation 2000). Tight correlations between egg area (applying image analysis) and egg mass as well as hatchling size confirm that this method provides a highly reliable measurement of egg size in *B. anynana* (Fischer *et al*. 2002). To calculate egg size for individual females, the mean across all measurement days was used as between-day variation in egg size was negligible (except for *experiment 4*, see below).

Statistical analysis

Data were analysed using one or two-way AN(C)OVAs. For two-way AN(C)OVAs, only significant interaction terms are given. Significant differences between treatment groups were located using Tukey's HSD. Effects of egg size and longevity on fecundity were investigated using multiple regressions (stepwise forward addition of variables, Ridge regression). Throughout all experiments, females laying less than 10 eggs during their life where excluded from subsequent analyses. All statistical tests were performed using Minitab (13.31) or Statistica (6.1). Throughout the text means are given ± 1 standard error.

Results

Experiment 1: Time series

Application of pyriproxyfen significantly affected lifetime fecundity (ANOVA: $F_{4,170}$ = 3.4, P = 0.011), egg-laying rate ($F_{4,170}$ = 6.7, P < 0.0001), and longevity ($F_{4,170}$ = 5.1, P = 0.001; Table 1) in female *B. anynana* butterflies. The groups being treated with pyriproxyfen on days 4 and 6 following adult eclosion (i.e. just before the onset of or during oviposition) showed increased fecundity (38 and 27 %, respectively, relative to controls) and egg-laying rates (91 and 56 %, respectively), whereas those being treated earlier were very similar to controls (Table 1).

Table 1. *Effects of pyriproxyfen (10 µg in 3 µl hexane; applied on day 0, 2, 4 or 6 following eclosion) on female Bicyclus anynana reproductive traits and longevity (means ± SE). Control females were treated with 3 µl hexane on day 0. Different superscript characters within columns indicate significant differences between treatment groups (Tukey HSD after ANOVA). Note the substantial increase in egg numbers in the group being treated with pyriproxyfen on day 4 of adult life.*

Treatment	Fecundity	Eggs day^{-1}	Egg size [mm^2]	Longevity [days]	*n*
Pyriproxyfen on day 0	89.6 ± 13.3^{a,b}	5.2 ± 0.69^{a}	0.628 ± 0.006^{a}	16.7 ± 0.93^{a,b}	27
Pyriproxyfen on day 2	79.3 ± 9.2^{a}	5.2 ± 0.56^{a}	0.629 ± 0.007^{a}	15.4 ± 0.57^{a,b}	34
Pyriproxyfen on day 4	136.2 ± 16.3^{b}	9.3 ± 0.92^{b}	0.621 ± 0.007^{a}	14.3 ± 0.66^{a}	36
Pyriproxyfen on day 6	115.4 ± 14.4^{a,b}	7.6 ± 0.85^{a,b}	0.624 ± 0.007^{a}	14.4 ± 0.65^{a}	37
Control (hexane)	84.2 ± 11.3^{a}	4.9 ± 0.85^{a}	0.622 ± 0.007^{a}	18.2 ± 0.94^{b}	37

Especially the group being treated on day 4 showed a marked increase in daily fecundity at the beginning of the oviposition period (Figure 1). On the other hand, pyriproxyfen seems to have detrimental effects on survival, as control animals tended to live longest (Table 1). Both groups achieving the highest fecundity and egg-laying rate, in contrast, tended to live shortest. Opposite to the above traits, egg size was not significantly affected by different treatments ($F_{4,170}$ = 6.20, P = 0.92). On the other hand, pyriproxyfen seems to have detrimental effects on survival, as control animals tended to live longest (Table 1). Both groups achieving the highest fecundity and egg-laying rate, in contrast, tended to live shortest. Opposite to the above traits, egg size was not significantly affected by different treatments ($F_{4,170}$ = 6.20, P = 0.92).

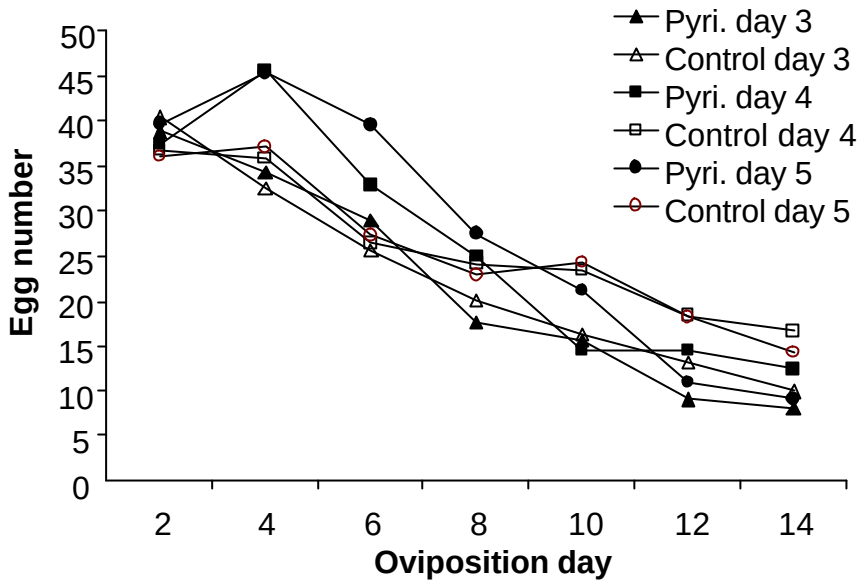

Figure 1. *Mean daily fecundity over time for groups of Bicyclus anynana females treated with 10 µg pyriproxyfen (Pyri.) in 3 µl hexane on day 0, 2, 4 or 6 following adult eclosion. Note that oviposition day 2 equals day 6 of adult life (see Methods). Control females were treated with 3 µl hexane on day 0. For clarity, no standard errors are given.*

As differences in mortality rates among groups may influence the results regarding reproductive traits (especially lifetime fecundity), these traits were reanalysed using the first 6 days of the oviposition period only. During this period mortality was low and the effects of hormonal treatments should be largest. These results, however, were not qualitatively different from the ones obtained before (fecundity: $F_{4,170} = 4.6$, $P = 0.002$; egg-laying rate: $F_{4,170} = 7.17$, $P < 0.0001$; egg size: $F_{4,170} = 0.31$, $P = 0.87$).

Experiment 2: Concentration series

Application of different concentrations of pyriproxyfen yielded significant effects on lifetime fecundity ($F_{3,141} = 5.5$, $P = 0.001$), egg-laying rate ($F_{3,141} = 3.2$, $P = 0.024$) and longevity ($F_{3,141} = 6.9$, $P < 0.001$; Table 2). Among the females that were treated with pyriproxyfen those receiving 1 µg pyriproxyfen had a higher fecundity (and tended to have a higher egg-laying rate) than hexane controls, whereas higher pyriproxyfen concentrations had no detectable effect. Longevity was significantly reduced in the group being treated with 100 µg pyriproxyfen (Figure 2).

Table 2. *Effects of different concentrations of pyriproxyfen in 3 µl hexane on female Bicyclus anynana reproductive traits and longevity (means ± SE). Control animals were treated with 3 µl hexane. All applications took place on day 4 of adult life. Different superscript characters within columns indicate significant differences between treatment groups (Tukey HSD after ANOVA).*

Treatment	Fecundity	Eggs day^{-1}	Egg size [mm^2]	Longevity [days]	*n*
1 µg pyriproxyfen	115.9 ± 9.7^{a}	6.7 ± 0.56^{a}	0.614 ± 0.007^{a}	17.8 ± 0.9^{a}	39
10 µg pyriproxyfen	68.6 ± 11.1^{b}	4.5 ± 0.64^{a,b}	0.615 ± 0.007^{a}	16.8 ± 1.0^{a}	30
100 µg pyriproxyfen	75.2 ± 10.5^{b}	5.0 ± 0.58^{a,b}	0.622 ± 0.007^{a}	13.1 ± 0.9^{b}	37
Control (hexane)	65.8 ± 10.0^{b}	4.5 ± 0.56^{b}	0.614 ± 0.007^{a}	18.7 ± 0.9^{a}	39

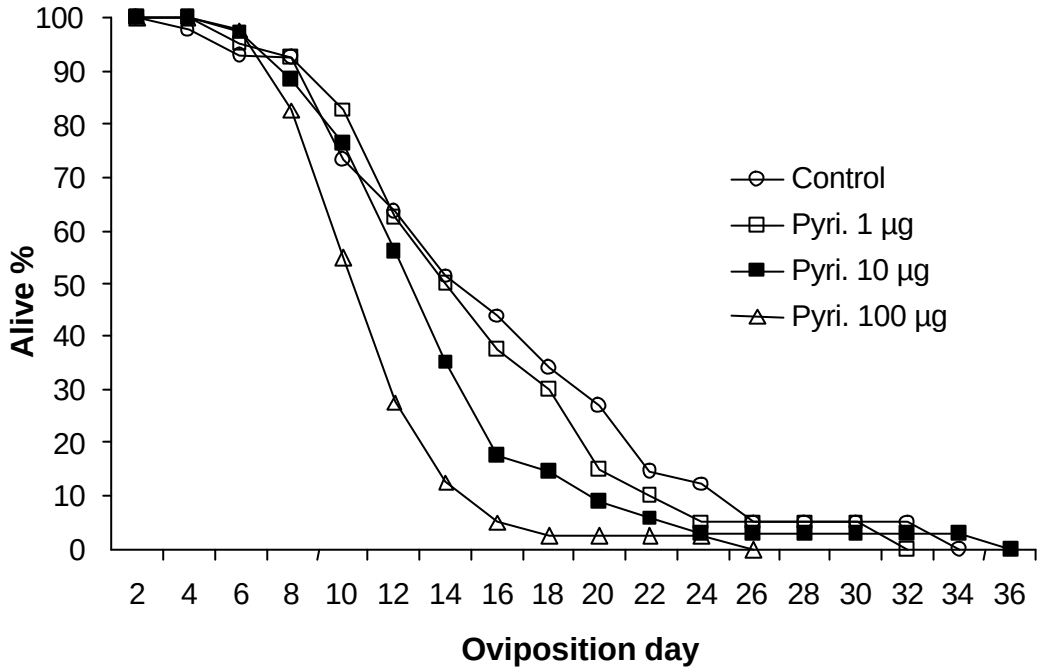

Figure 2. *Cumulative survival probabilities of female Bicyclus anynana butterflies after treatment with 1, 10 or 100 µg pyriproxyfen (Pyri.) in 3 µl hexane on day 4 of adult life. Oviposition day 2 equals day 6 of adult life (see Methods). The control group received 3 µl pure hexane on day 4.*

As in the first experiment, egg size was not affected by pyriproxyfen treatment ($F_{3,141}$ = 0.31, P = 0.82).

Table 3. *Effects of repeated applications of pyriproxyfen in 3 µl acetone on female Bicyclus anynana reproductive traits and longevity (means ± SE). Control females were treated with 3 µl acetone. All applications took place on seven consecutive days (days 0 to 6 of adult life). Different superscript characters within columns indicate significant differences between treatment groups (Tukey HSD after ANOVA).*

Treatment	Fecundity	Eggs day^{-1}	Egg size [mm^2]	Longevity [days]	*n*
1 µl pyriproxyfen	67.3 ± 5.7^{a}	5.6 ± 0.40^{a}	0.646 ± 0.005^{a}	12.1 ± 0.46^{a}	83
5 µl pyriproxyfen	60.1 ± 6.0^{a}	5.2 ± 0.42^{a}	0.654 ± 0.005^{a}	11.5 ± 0.48^{a}	75
Control (acetone)	79.6 ± 5.8^{a}	5.8 ± 0.41^{a}	0.639 ± 0.005^{a}	13.8 ± 0.46^{b}	80

Experiment 4: 'A closer look'

Pyriproxyfen and application day significantly affected lifetime fecundity (two-way ANOVAs; pyriproxyfen: $F_{1,420} = 7.0$, $P = 0.009$; application day: $F_{2,420} = 4.7$, $P = 0.010$), egg-laying rate (pyriproxyfen: $F_{1,420} = 4.0$, $P = 0.045$; application day: $F_{2,420} = 3.9$, $P = 0.022$) and reproductive investment (pyriproxyfen: $F_{1,420} = 11.1$ $P < 0.001$; application day: $F_{2,420} = 4.3$, $P = 0.014$). Egg size (pyriproxyfen: $F_{1,420} = 11.3$, $P < 0.001$; application day: $F_{2,420} = 0.9$, $P = 0.43$) and longevity (pyriproxyfen: $F_{1,420} = 87.8$, $P < 0.001$; application day: $F_{2,420,} = 1.7$, $P = 0.19$), however, were affected by pyriproxyfen only (Table 4).

Table 4. *Effects of pyriproxyfen (5 µg in 3 µl acetone; applied on day 3, 4 or 5 following eclosion) on female Bicyclus anynana reproductive traits and longevity (means ± SE). Control females were treated with 3 µl acetone on day 3, 4 or 5. Different superscript characters within columns indicate significant differences between treatment groups (Tukey HSD after ANOVA).*

Treatment	Fecundity	Eggs day^{-1}	Egg size [mm^2]	Longevity [days]	Repr. Invest. [mg]	*n*
Pyriproxyfen on day 3	133.5 ± 11.4^{a}	8.4 ± 0.6^{a,b}	0.633 ± 0.006^{a,b}	16.0 ± 0.67^{a}	50.8 ± 4.3^{a}	70
Control day 3	163.8 ± 12.9^{a,b}	7.8 ± 0.5^{a}	0.649 ± 0.005^{a}	20.8 ± 0.90^{b}	64.2 ± 4.9^{a,b}	73
Pyriproxyfen on day 4	166.2 ± 12.9^{a,b}	10.0 ± 0.7^{a,b}	0.626 ± 0.004^{b}	16.5 ± 0.50^{a}	62.7 ± 4.7^{a,b}	73
Control day 4	198.0 ± 13.0^{b}	9.0 ± 0.5^{a,b}	0.647 ± 0.004^{a,b}	22.3 ± 0.80^{b}	78.4 ± 5.1^{b}	67
Pyriproxyfen on day 5	173.1 ± 12.6^{a,b}	11.1 ± 1.0^{b}	0.624 ± 0.005^{b}	16.4 ± 0.62^{a}	64.8 ± 4.5^{a,b}	72
Control day 5	194.1 ± 12.8^{b}	8.9 ± 0.5^{a,b}	0.641 ± 0.005^{a,b}	22.0 ± 0.86^{b}	75.4 ± 4.9^{b}	72

Generally, the pyriproxyfen treated groups laid fewer eggs (158 ± 7 versus 185 ± 7 eggs) of reduced size (0.628 ± 0.004 versus 0.646 ± 0.004 mm^2; concomitantly reducing reproductive investment: 59.8 ± 2.8 versus 72.6 ± 2.8 mg), but had increased egg-laying rates (9.7 ± 0.4 versus 8.7 ± 0.4 eggs/day) compared to control groups. As, however, longevity was also shorter in hormone-treated animals (16.2 ± 0.4 versus 21.2 ± 0.4 days) which may affect lifetime fecundity and reproductive investment, these data were reanalyzed including longevity as covariate in the models. The ANCOVAs showed that, when correcting for differences in longevity, the effects of pyriproxyfen were not significant anymore (fecundity: pyriproxyfen $F_{1,419}$ = 1.4, P = 0.249; application day $F_{2,419}$ = 3.4, P = 0.033; longevity $F_{1,419}$ = 84.3, P < 0.0001; reproductive investment: pyriproxyfen $F_{1,419}$ = 0.20, P = 0.67; application day $F_{2,419}$ = 3.1, P = 0.047; longevity $F_{1,419}$ = 80.6, P < 0.0001). These findings suggest that the higher lifetime fecundity and reproductive investment in the controls is due to their longer life.

Accordingly, multiple regressions revealed positive correlations between lifetime fecundity and longevity throughout all treatment groups (r^2_{mult}: 13-32 %; Table 5). Further, such analyses indicated negative correlations between lifetime fecundity and egg size (r^2_{mult}: 15-25 %; except for day 4 controls), suggesting a trade-off between egg size and number. In four groups, longevity is the most important predictor of lifetime fecundity followed by egg size. The relative importance of these two variables, however, is reversed in the remaining two groups (pyriproxyfen day 4 and 5; Table 5). Interestingly, the latter comprise the groups with highest egg-laying rates (Table 4). The general patterns of positive relationships between fecundity and longevity and negative ones between fecundity and egg size were also found in the other experiments (data not shown).

However, even though application of pyriproxyfen tended to reduce lifetime fecundity, daily fecundity was significantly higher in pyriproxyfen-treated females compared to controls during oviposition days 4-6 (i.e. shortly after hormone applications; Table 6), while this pattern changed from oviposition day 10 onwards (Figure 3). Regarding daily egg sizes, the treatment effect was visible from oviposition day 4 until the end of the oviposition period (Figure 4).

As indicated by the significant effects of the application day (see above), treating the females on day 3 of adult life significantly decreased performance as compared to application days 4 or 5 (fecundity day 3: 150 ± 9, day 4: 181 ± 9, day 5: 184 ± 9 eggs; egg-laying rate day 3: 8.3 ± 0.4, day 4: 9.5 ± 0.4, day 5: 9.9 ± 0.4 eggs/day; reproductive investment: day 3: 58.3 ± 3.4, day 4: 69.9 ± 3.4, day 5: 70.4 ± 3.4 mg).

Table 5. *Results of multiple regressions (stepwise forward addition of variables; Ridge regression; lambda = 0.10; F > 1.0 for inclusion) for the effects of longevity and mean egg size on fecundity. Treatment groups refer to females being treated with 5 µg pyriproxyfen in 3 µl acetone or 3 µl pure acetone (controls) on day 3, 4 or 5 following eclosion. Given are standardized partial regression coefficients Beta (standard error in parentheses), multiple coefficients of determination r^2_{mult}, F-value and significance level. P < 0.05 in bold.*

Treatment	Predictor	Beta	r^2_{mult}	F	n	P
Pyriproxyfen on day 3	Longevity	0.331 (0.111)	0.142	11.12	69	**< 0.001**
	Egg size	-0.127 (0.111)	0.159	1.31		0.256
Control day 3	Longevity	0.410 (0.102)	0.215	19.45	73	**< 0.001**
	Egg size	-0.146 (0.102)	0.237	2.05		0.156
Pyriproxyfen on day 4	Egg size	-0.326 (0.101)	0.149	12.43	73	**< 0.001**
	Longevity	0.277 (0.101)	0.231	7.51		**0.008**
Control day 4	Longevity	0.346 (0.110)	0.132	9.85	67	**0.003**
Pyriproxyfen on day 5	Egg size	-0.367 (0.099)	0.222	19.91	72	**< 0.001**
	Longevity	0.302 (0.099)	0.315	9.40		**0.003**
Control day 5	Longevity	0.343 (0.099)	0.150	12.35	72	**< 0.001**
	Egg size	-0.299 (0.099)	0.248	8.89		**0.004**

Next page (page 94): **Table 6.** *Effects of pyriproxyfen (5 µg in 3 µl acetone; applied on day 3, 4 or 5 following eclosion) on female Bicyclus anynana early fecundity (first eight oviposition days; means ± SE). Control females were treated with 3 µl acetone on day 3, 4 or 5. On days 8 and 10 (i.e. ca. 3-6 days after pyriproxyfen applications), fecundity was significantly higher in hormone-treated females (two-way ANOVAs on daily fecundity; effects of pyriproxyfen application).*

Table 6.

Treatment	Day 2	Day 4	Day 6	Day 8
Pyriproxyfen on day 3	38.8 ± 3.5	34.3 ± 3.3	28.9 ± 2.7	17.6 ± 2.1
Control day 3	40.4 ± 3.3	32.6 ± 2.6	25.6 ± 2.3	20.1 ± 2.1
Pyriproxyfen on day 4	37.4 ± 3.2	45.6 ± 3.6	32.9 ± 3.1	25.1 ± 2.8
Control day 4	36.6 ± 3.1	35.8 ± 3.0	26.4 ± 2.4	24.1 ± 2.1
Pyriproxyfen on day 5	39.6 ± 3.0	45.4 ± 3.7	39.5 ± 3.7	27.5 ± 2.6
Control day 5	35.9 ± 3.1	37.0 ± 2.7	27.3 ± 2.6	22.9 ± 2.0
ANOVA	$F_{1,420} = 0.25$	$F_{1,415} = 6.86$	$F_{1,402} = 9.79$	$F_{1,376} = 0.30$
	$P = 0.62$	$P = 0.0091$	$P = 0.0019$	$P = 0.59$

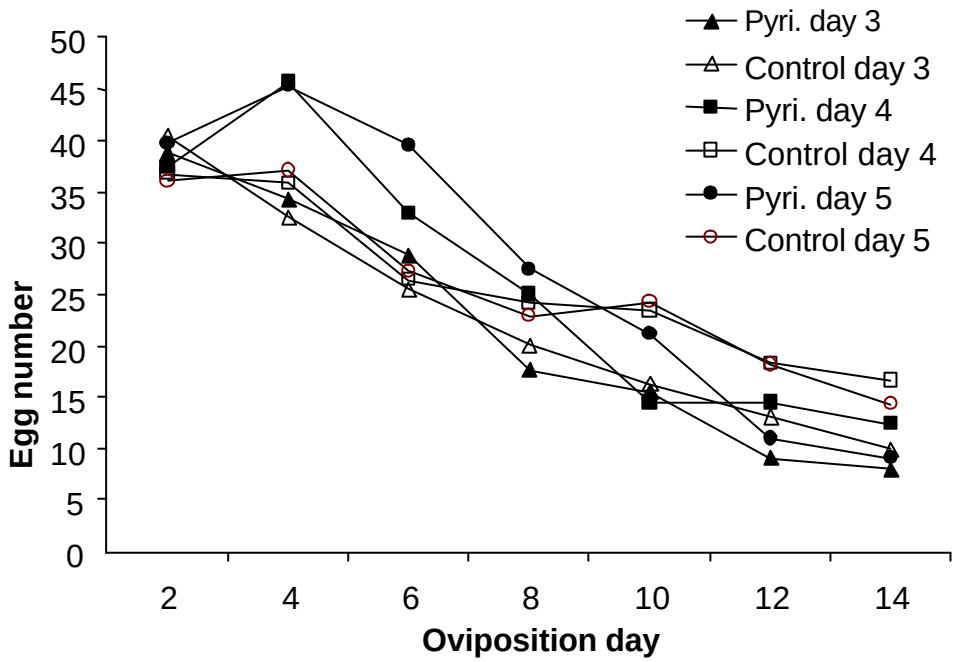

Figure 3. *Mean daily fecundity over time for groups of Bicyclus anynana females treated with 5 µg pyriproxyfen in 3 µl acetone on day 3, 4 or 5 following adult eclosion. Oviposition day 2 equals day 6 of adult life (see Methods). Controls were treated with 3 µl pure acetone the same days. While pyriproxyfen and control groups do not differ on oviposition day 2, the former lay significantly more eggs than controls on days 4 to 6 (see Table 6). From day 10 onwards, however, the pattern is reversed with controls laying significantly more eggs (two-way ANOVAs on daily fecundity, effects of pyriproxyfen application, all P-values < 0.02). For clarity, no standard errors are given.*

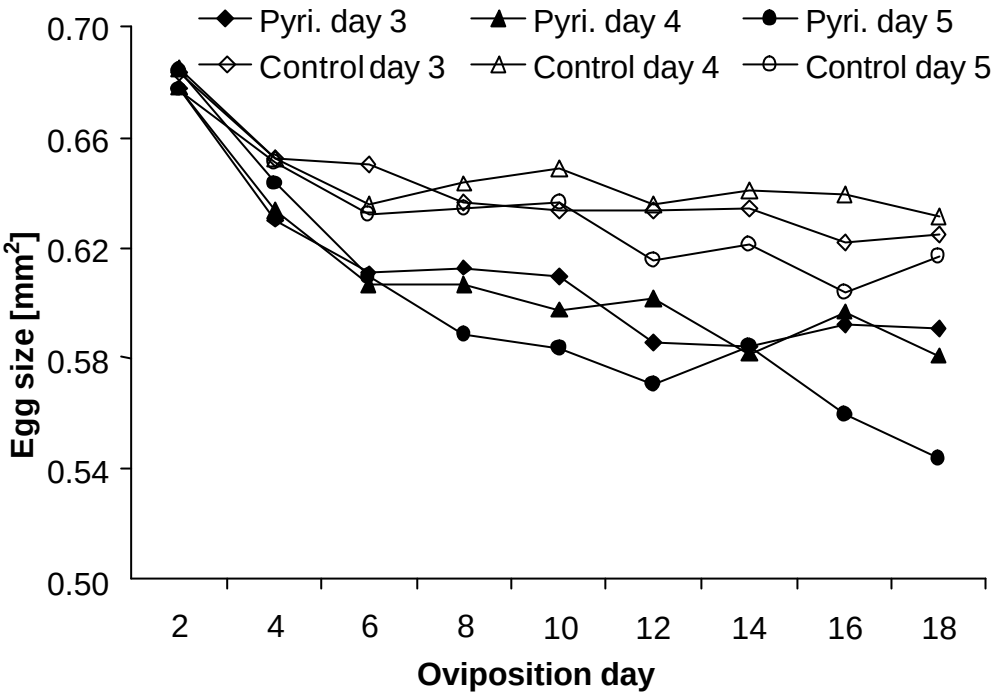

Figure 4. *Mean daily egg size over time for groups of Bicyclus anynana females treated with 5 µg pyriproxyfen in 3 µl acetone on day 3, 4 or 5 following adult eclosion. Oviposition day 2 equals day 6 of adult life (see Methods). Controls were treated with 3 µl pure acetone the same days. Except for oviposition day 2, egg size was significantly smaller in pyriproxyfen than in control groups (two-way ANOVAs on daily egg sizes, effects of pyriproxyfen application, all P-values < 0.001).*

Discussion

When applied at the beginning of the oviposition period (days 4 or 5 of adult life), pyriproxyfen did affect reproductive output and longevity in female *B. anynana* butterflies. Earlier (days 0 and 2 of adult life) applications, however, showed no effect on reproductive traits. Likewise, applications on day 6 of adult life yielded weaker responses compared to applications on day 4 (cf. Table 1). These findings indicate that females are most sensitive to pyriproxyfen at the onset of oviposition, coinciding with naturally increasing JH titres and intensive vitellogenin synthesis in other Lepidoptera (e.g. Cusson *et al*. 1994; Ramaswamy *et al*. 1997; Zeng *et al*. 1997; Range *et al*. 2002).

Apart from application day, concentrations influenced the response to pyriproxyfen. High concentrations (100 µg pyriproxyfen) as well as repeated applications of smaller

amounts did not change reproductive traits, but tended to reduce longevity (Tables 2 and 3). In insects, the amount of free JH is usually controlled by JH specific esterases and some other mechanisms (Gäde *et al*. 1997; Gilbert *et al*. 2000; Kamita *et al*. 2003). However, applying very high concentrations or repeated applications of pyriproxyfen may defeat the regulating mechanisms, resulting in detrimentally high JH titres inducing pharmacological effects (e.g. Webb *et al*. 1999; Wilson 2004). Furthermore, there is evidence that high doses of pyriproxyfen may act antagonistically, thereby decreasing vitellogenin synthesis (Edwards *et al*. 1993). Such effects are presumably causing the lack of a response to pyriproxyfen in *experiment 3* (repeated applications).

Regarding the effects of lower amounts of pyriproxyfen, it is intriguing that 10 µg did affect reproduction in *experiment 1*, while this was not the case in *experiment 2* (despite using the same solvent and application day). In the latter, in contrast, 1 µg pyriproxyfen (a concentration not used before) yielded positive responses. Though we cannot really explain the difference among experiments (note that both experiments used the same experimental conditions e.g. with regard to temperature, relative humidity, light cycle), environmental differences are likely to be involved. In general, the outcome of biological experiments can be largely affected by assay conditions (e.g. Rose 1984; Leroi *et al*. 1994; Ackermann *et al*. 2001). In our case, such differences could have been mediated through changes in food quality and/or quality. Such effects may easily affect JH titres (which depend at least partly on environmental conditions; Ramaswamy *et al*. 1997; Yin *et al*. 1999; Trumbo & Robinson 2004) as well as butterfly size (Boggs & Ross 1993; Fischer & Fiedler 2001; Bauerfeind & Fischer 2005a,b). Consequently, the same amount of pyriproxyfen applied to animals differing in JH haemolymph titres or body mass may have strikingly different implications. Note that no mass measures of butterflies were taken, and that concentrations were not adjusted to a fixed amount per standard mass. Nevertheless, our experiments show that, for 'average' *B. anynana* butterflies, amounts between 1 and 10 µg pyriproxyfen should usually work. This is confirmed by our last experiment using 5 µg pyriproxyfen (cf. Table 4).

Thus, provided that an appropriate amount of pyriproxyfen is applied during the sensitive period as outlined above, it consistently increased egg-laying rate and

concomitantly fecundity in *B. anynana* females across experiments. The latter effect, however, was transient and restricted to a couple of days following the application (i.e. to the beginning of the oviposition period in our experiments; cf. Figures 1 and 3; Table 6). This pattern matches closely with the fact that effects of pyriproxyfen have a delay of around 24 hours only (Edwards *et al*. 1993), and that JH mimics degrade quite rapidly (Gilbert *et al*. 2000; Kamita *et al*. 2003). Comparable results were found in some other insects (fecundity in *Heliothis virescens*, Ramaswamy *et al*. 1997; ovarian mass in *Gryllus assimilis*, Zera *et al*. 1998; fecundity and egg-laying rate in *Nicrophorus* spp., Trumbo & Robinson 2004).

However, the increase in early fecundity was accompanied by a decrease in longevity throughout (cf. Herman & Tatar 2001; Tatar & Yin 2001; Trumbo & Robinson 2004). As egg production is an energetically demanding process, the increase in early fecundity may cause an earlier depletion of resources and may thereby reduce longevity, thus demonstrating a frequently anticipated trade-off between early reproduction and longevity (Reznick *et al*. 2000; Barnes & Partridge 2003; Yanagi & Miyatake 2003). Empirical work, especially on *Drosophila*, has provided evidence that one cost of reproduction is acceleration of the rate of ageing (Barnes & Partridge 2003) and that JHs are involved in this process (Herman & Tatar 2001; Tatar *et al*. 2001, 2003; but see Richards *et al*. 2005).

Such a trade-off can be expected especially in species like *Bicyclus* butterflies, which draw heavily from nitrogenous reserves accumulated during the larval stage for reproduction and general maintenance (e.g. Boggs 1997; Fischer *et al*. 2004; O'Brien *et al*. 2004). However, competition for shared energy reserves and an increased allocation of these resources to reproduction alone cannot explain all of our results, as in *experiment 4* pyriproxyfen-treated females had reduced longevity in spite of a lower lifetime fecundity (Table 4). Nevertheless, these females had a significantly higher egg-laying rate throughout. Thus, the presumed trade-off appears to be more complex and possibly occurs between longevity and mobilisation/acquisition of energy per time unit rather than between longevity and the amount of energy allocated to reproduction *per se*.

In contrast to early fecundity, lifetime fecundity was not consistently higher in pyriproxyfen-treated females, but was even reduced compared to control females in *experiment 4* (contrasting results of *experiments 1* and *2*). At the same time, longevity and lifetime fecundity was much higher in *experiment 4* compared to the earlier ones, probably as a result of changing the solvent from hexane to acetone. Thus, when given enough time, control females may eventually outperform hormone-treated females by maintaining higher oviposition rates late in life and by having a prolonged oviposition period (Figure 4). This interpretation is supported by ANCOVA analyses. When differences in longevity are controlled for, the differences in lifetime fecundity across treatment groups disappear. Also in multiple regressions, longevity was the most important predictor of lifetime fecundity, followed by egg size.

The reduced lifetime fecundity (and egg size; see below) in pyriproxyfen-treated females in *experiment 4* also caused a significantly reduced reproductive investment compared to control females. As discussed above, this could be the result of a more 'intense' life with higher egg production rates earlier on (Reznick *et al*. 2000; Barnes & Partridge 2003). It should be noted, though, that this does not mean that increased levels of JH lower overall fitness. Under natural conditions, where random mortality occurs, total egg number and reproductive investment is unlikely to be a good estimator of fitness. The ability to lay as many eggs as possible within a short period of time at the beginning of oviposition should be much more important, as the first eggs laid tend to be the most important ones in terms of fitness (Begon & Parker 1986). The time of application also seems to be important. In *experiment 4*, treating the females on day 3 of adult life significantly decreased fecundity, egg-laying rate and reproductive investment as compared to application days 4 or 5 (Table 4).

In contrast to egg-laying rate and early fecundity, egg size was affected by pyriproxyfen in *experiment 4* only. This difference to the other experiments could result from using a different solvent (acetone) and the (concomitantly) almost twofold increase in lifetime fecundity, and to a smaller extent from increased statistical power due to large sample sizes. Thus, the frequently assumed and often found trade-off between egg size and egg number (Smith & Fretwell 1974; Fox & Czesak 2000; Roff 2002) may be obscured when the number of eggs laid per day is relatively low. In such a situation, females may not be forced to reduce egg size in order to lay larger

egg numbers. This notion is supported by the fact that in the groups showing the highest egg-laying rates, egg size but not longevity is the most important predictor of lifetime fecundity (Table 5).

In summary, our experiments showed that application of pyriproxyfen consistently increased early fecundity and egg-laying rate in *B. anynana* females despite possible pharmacological effects. These increases were accompanied by a reduction in adult life span, thus potentially demonstrating a JH-mediated trade-off between present and future reproduction. Egg size, in contrast, was not consistently affected, but decreased in the last experiment only. Further, pyriproxyfen action was restricted to certain sensitive periods, and high pyriproxyfen concentrations yielded detrimental effects (e.g. McNeil 1975; Nijhout 1983; Emlen & Nijhout 1999; Webb *et al*. 1999; Moczek & Nijhout 2002).

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6.2. Ovarian dynamics, egg size and egg number in relation to temperature and mating status in a butterfly

Marc J. Steigenga[1]*, Klaus Fischer[1]

[1] *Department of Animal Ecology I, Bayreuth University, D-95440 Bayreuth, Germany*

***Author for correspondence:**

Marc Steigenga
Department of Animal Ecology I
Bayreuth University
P.O. Box 101 251
D-95440 Bayreuth, Germany

Tel.: +49-921-553079
Fax: +49-921-552784
E-mail: marc.steigenga@uni-bayreuth.de

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Abstract

Although the temperature-size rule, i.e. an increase in egg (and body) size at lower temperatures, applies almost universally to ectotherms, the developmental mechanisms underlying this consistent pattern of phenotypic plasticity are hitherto unknown. By investigating ovarian dynamics and reproductive output in the tropical butterfly, *Bicyclus anynana* (Butler 1879) (Lepidoptera: Nymphalidae, Satyrinae), in relation to oviposition temperature and mating status, we test the relevance of several competing hypotheses for temperature-mediated variation in egg size and number. As expected, females ovipositing at a lower temperature laid fewer but larger eggs compared to those ovipositing at a higher temperature. Despite pronounced differences in egg-laying rates, oocyte numbers were equal across temperatures at any given time, while oocyte size increased at the lower temperature. Mating, in contrast, greatly reduced oocyte numbers in mated as compared to virgin females. Our results indicate that temperature-mediated plasticity in egg size can neither be explained by variation in body size (all butterflies were reared in a common environment), by reduced costs of somatic maintenance at lower temperatures enabling the allocation of more resources to reproduction (reproductive investment was higher at the higher temperature), nor by delayed oviposition (no accumulation of oocytes at the lower temperature, in contrast to virgin females). Rather, low temperatures greatly reduced oocyte production (i.e. differentiation) rate and probably prolonged egg maturation time, causing low egg-laying rates. Our data thus suggest that oocyte growth is less sensitive to temperature than is oocyte production, resulting in a lower number of larger eggs at lower temperatures.

Introduction

Egg size is a most interesting life history trait as it is both, a maternal and a progeny character, and as it is generally believed to be closely related to fitness (Fox & Czesak 2000; Fischer *et al*. 2003a). Consequently, the study of how mothers allocate their resources to offspring number and size has provided a key area of life-history research (Smith & Fretwell 1974; Bernardo 1996a,b; Fox & Czesak 2000; Roff 2002; Fischer *et al*. 2006a). Despite much effort over recent decades, however, we still have a rather incomplete understanding of the causes and consequences of variation in offspring size (Einum & Fleming 2000; Fox & Czesak 2000; Fischer *et al*. 2003a,

2006a). One of the most striking and best-described phenomena with regard to variation in insect egg size is temperature-mediated plasticity. Eggs of ectothermic animals were commonly found to be larger in colder regions and at colder times, and under laboratory conditions females usually lay larger eggs at lower temperatures (e.g. Azevedo *et al.* 1996; Crill *et al*. 1996; Yampolski & Scheiner 1996; Ernsting & Isaaks 1997; Blanckenhorn 2000; Atkinson *et al.* 2001; Fischer *et al*. 2003a, 2006b). This almost universal pattern of larger body, egg, or cell size at lower temperatures is usually referred to as the temperature-size rule (Atkinson 1994; Atkinson *et al*. 2001). However, we do not yet understand the underlying mechanisms shaping this consistent pattern of phenotypic plasticity (Azevedo *et al*. 1996; Crill *et al*. 1996; Blanckenhorn 2000; Fox & Czesak 2000; Fischer *et al*. 2003a; Walters & Hassall 2006).

Potential mechanisms underlying temperature-mediated variation in egg size include: (1) a larger body size at lower temperatures resulting in larger eggs (Moore & Folt 1993; but Avelar 1993; Azevedo *et al*. 1996; Ernsting & Isaaks 1997; Fischer *et al*. 2003a); (2) reduced costs of somatic maintenance and concomitantly a higher proportion of resources available for egg production (Avelar 1993; Fox & Czesak 2000; but Ernsting & Isaaks 2000; Fischer *et al*. 2003a); 3) a change of oocyte production rate relative to oocyte growth (vitellogenesis), affecting both number and size of eggs (Van der Have & De Jong 1996; Van Voorhies 1996; Ernsting & Isaaks 1997, 2000; Blanckenhorn & Henseler 2005); and (4) an increased oocyte growth due to delayed oviposition (Wallin *et al*. 1992; Huey *et al*. 1995; but Fox *et al.* 1997; Fox & Czesak 2000).

Here we set out to test the relevance of these competing hypotheses for variation in egg size (and number) in the tropical butterfly *Bicyclus anynana* (Butler 1879) (Lepidoptera: Nymphalidae, Satyrinae). Therefore, we analyzed ovarian dynamics in addition to egg size and number in female butterflies divided among two oviposition temperatures, but reared in a common environment. Hypotheses (1) and (2) appear rather unlikely, as differences in egg size have been repeatedly shown to be independent of variation in body size, amongst others as they can be induced in the adult stage (Fischer *et al*. 2003a,b,c, 2004, 2006b; Steigenga *et al*. 2005), and as reproductive investment was found to increase rather than decrease at higher

temperatures (Ernsting & Isaaks 1997, 2000; Fischer *et al*. 2003a). To facilitate the distinction between hypotheses (3) and (4) we also included an experiment manipulating mating status (virgin versus once mated) along with temperature. Mating is known to stimulate oviposition in *B. anynana* and other insects, and possibly also affects egg production (Chapman *et al*. 1998, 2001; Fischer in review). In the absence of such oviposition stimuli, eggs are expected to accumulate in the oviducts (e.g. Sadeghi & Gilbert 2000; Roberts & Schmidt 2004).

To investigate temperature effects on egg size, *B. anynana* appears to be a particularly suitable model organism, as environmentally-induced differences in reproductive strategies exist. Recent studies showed that females kept at a lower oviposition temperature laid larger but fewer eggs than those kept at a higher temperature (Fischer *et al*. 2003a,c). Based on differential survival probabilities among temperatures, these data suggest that it is profitable to produce fewer but larger offspring (with increased fitness) at a lower temperature, but more and smaller offspring at a higher temperature where offspring survival is generally high in this tropical butterfly (Fischer *et al.* 2003a,c). Consequently, females should benefit from adjusting the size of their eggs to the temperature experienced during oviposition, providing a predictable cue for the environmental conditions experienced by the offspring in early life. These suppositions closely match environmental demands, as *B. anynana* lives in a seasonal environment with a beneficial wet season of high temperatures, and a rather adverse dry season when average temperature is low (Brakefield 1997). This adaptive scenario is further supported by the existence of genetic variation in the temperature reaction norms, indicating potential for short term evolutionary change (Fischer *et al*. 2004, 2006b; Steigenga *et al*. 2005).

In this paper we investigate ovarian dynamics and reproductive output in relation to temperature and mating status, in order to unravel the hitherto unknown mechanisms underlying temperature-mediated plasticity in insect egg size. Specifically, we address the following issues: (1) does temperature affect oocyte production rates (i.e. differentiation), vitellogenesis (oocyte growth) and choriogenesis (egg shell formation) and what are its implications for the temperature-size rule? (2) Does mating stimulate oocyte production and / or oviposition?

Materials and methods

Study organism

Bicyclus anynana is a tropical, fruit-feeding butterfly with a distribution ranging from Southern Africa to Ethiopia, which feeds on a variety of fallen and decaying fruit (Larsen 1991; Brakefield 1997). The species exhibits striking phenotypic plasticity (two seasonal morphs) as an adaptation to alternative wet-dry seasonal environments and the associated changes in resting background and predation (Brakefield 1997; Lyytinen *et al*. 2004). Reproduction is essentially confined to the warmer wet season when oviposition plants are abundantly available, and where 2-3 generations occur. During the colder dry season reproduction ceases and butterflies do not mate before the first rains at the beginning of the next wet season (Windig 1994; Brakefield 1997).

A laboratory stock population was established at Bayreuth University, Germany, in 2003 from several hundred individuals derived from a well-established stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from over 80 gravid females caught at a single locality in Malawi. Several hundred adults are reared in each generation, maintaining high levels of heterozygosity at neutral loci (Van't Hof *et al*. 2005). For this study butterflies from the Bayreuth stock population were used.

General rearing

Throughout, all butterflies were reared in a climate room at 27°C, high relative humidity (70 %), and a photoperiod of L12:D12, thus mimicking wet season conditions. Larvae were fed on young maize plants and kept in population cages (50 x 50 x 80 cm). The resulting pupae were collected from the plants daily and transferred to cylindrical hanging cages. Throughout all experiments, butterflies had access to moist banana for adult feeding.

Experiment 1

Following adult eclosion, females were kept separate from males for two days, after which an equal number of random males was added to the females' cage for mating. After a mating period of two days, females were placed individually in labeled 1 L plastic containers with a fresh maize leaf for oviposition (replaced every other day).

Thereafter, females were randomly divided among 20°C and 27°C to induce a plastic response in egg size. Eggs were collected, counted and their size was measured (see below) on a bi-daily basis. At days 0, 3, 6, 9 and 12 after the division among temperatures, random samples of females were frozen and their ovaries were subsequently dissected in ice cold saline 1.15% (130mM NaCl, 5 mM KCl, 2mM $CaCl_2$, 50 mM Na_2HPO_4; PH = 7). In *B. anynana*, a clear plastic response in egg size in relation to temperature can be expected within this period (Fischer *et al.*, 2003a,b,c). As all insects, *B. anynana* females have two ovaries, each consisting of multiple ovarioles with numerous oocytes (Ziegler & Van Antwerpen 2006).

Following dissection, ovaries were stained for 60 minutes at room temperature using 1% Trypan Blue dye in 1.15% saline, after which excess stain was washed of with saline (cf. Shu *et al*. 1997; Webb *et al*. 1999). Oocytes were defined chorionated when not staining blue in the presence of Trypan Blue (appearing white and rather shiny), while unchorionated oocytes stained blue (Telfer & Anderson 1968; Webb *et al*. 1999). The number of chorionated and unchorionated oocytes was counted using a binocular (x8 magnification); the size of a random sample of chorionated oocytes was measured as outlined below. As this method yielded, unexpectedly, no significant differences in oocyte size across temperatures, another cohort consisting of higher sample size was reared and treated in the same manner as described above. All these females were frozen and their ovaries were dissected on day 12 after the division among temperatures. Here, only the size of the three largest (i.e. terminal) oocytes was measured.

Experiment 2

On day 2 after adult eclosion, females were randomly divided into two groups, one of which was allowed to mate with random males and was later provided with an oviposition plant, while the other was kept virgin and never received any oviposition substrate. Mating cages were continuously observed and mating couples were isolated. On day four of adult life, both mating groups were once again divided among 20°C and 27°C, resulting in four treatment groups. After 12 days at different temperatures, all females were frozen, their ovaries were dissected and stained, the number of chorionated and unchorionated oocytes was counted, and the size of the three largest chorionated oocytes was measured (see below).

Egg and oocyte measurement

As the eggs of *B. anynana* are nearly perfect spheres, their size was measured as cross-sectional projections (mm^2) using a digital camera (Leica DC300, Leica Microsystems, Wetzlar, Germany) connected to a stereo microscope (correlation between egg area and egg mass $r \sim 0.92$; Fischer *et al.*, 2002). The resulting images were automatically analysed using Scion Image public software (Scion Corporation 2000). For oocytes, which were often still incorporated in the ovaries, also digital images were captured, on which oocyte diameters were measured using Scion Image. To calculate egg and oocyte size for individual females, the mean of at least 10 eggs or chorionated oocytes (if possible) or the mean of the largest three chorionated oocytes was used (see above).

Statistical analysis

Data were analysed using analyses of variance (ANOVAs) with oviposition or dissection day, temperature and mating status (if applicable) as fixed factors. All statistical tests were performed using Minitab (13.31) or Statistica (6.1). Throughout the text means are given ± 1 SEM. As oocyte size and number were not normally distributed, such data were LN (*experiment 1*) or square root transformed (*experiment 2*) to meet ANOVA requirements.

Results

Experiment 1

Egg number

At 20°C, fecundity was much reduced compared to 27°C (102.5 ± 10.8 vs. 199.1 ± 11.4 eggs), especially at the beginning of the oviposition period (Figure 1a; Table 1). Bi-daily fecundity peaked at oviposition day 4, showing afterwards a steady decline. The significant temperature by oviposition day interaction results from the steeper decline in egg numbers at 27°C compared to 20°C. Regarding egg size, females transferred to 20°C laid significantly larger eggs than those transferred to 27°C from oviposition day 4 onwards (Figure 1b; Table 1).

Despite the significant variation in egg numbers across temperatures, numbers of chorionated and unchorionated oocytes as well as their percentages did not differ between 20 and 27°C (Figures 2a-c; Table 1). Unchorionated oocytes showed highest numbers at the beginning of the oviposition period, dropping afterwards to a fairly constant level of 4-6 oocytes. Chorionated oocytes, in contrast, reached highest numbers on oviposition days 3-6 Concomitantly, relative values for chorionated oocytes were highest on oviposition day 6. Oocyte size did not differ significantly across temperatures, but tended to increase with time (Figure 2d). In the additional cohort of females dissected on day 12 after the division among temperatures, the size of the terminal oocytes was significantly larger at 20°C than at 27°C (0.692 mm^2 ± 0.006, n = 95 vs. 0.653 mm^2 ± 0.006, n = 87; t-test: t = -4.6, P < 0.0001).

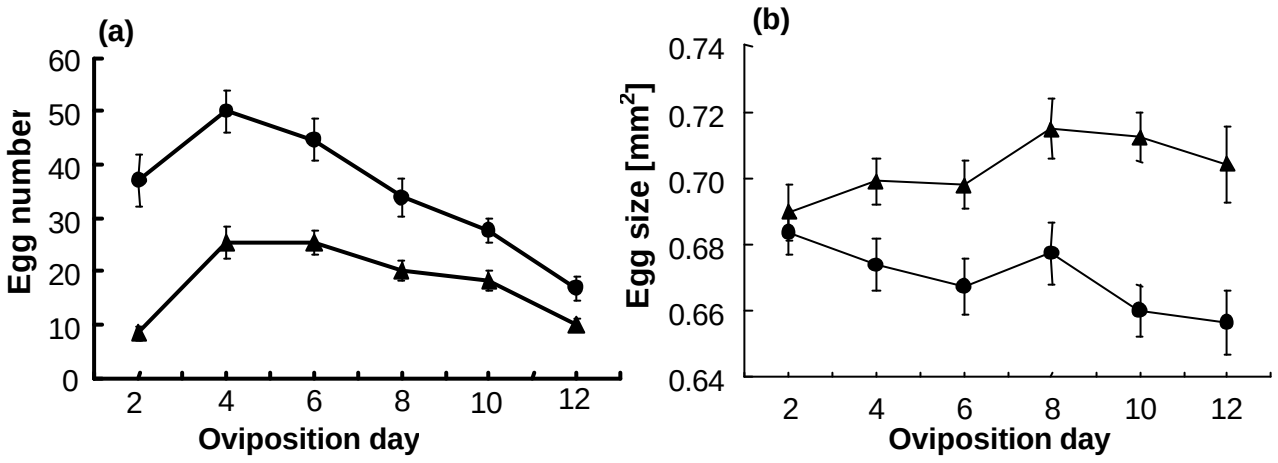

Figure 1*: Bi-daily fecundity (a) and egg size (b) over time for Bicyclus anynana females ovipositing at 20°C or 27°C. Oviposition day 2 equals day 6 of adult life. Given are means ± 1SEM. Triangles: 20°C, circles: 27°C.*

Concomitantly, relative values for chorionated oocytes were highest on oviposition day 6. Oocyte size did not differ significantly across temperatures, but tended to increase with time (Figure 2d). In the additional cohort of females dissected on day 12 after the division among temperatures, the size of the terminal oocytes was significantly larger at 20°C than at 27°C (0.692 mm^2 ± 0.006, n = 95 vs. 0.653 mm^2 ± 0.006, n = 87; t-test: t = -4.6, P < 0.0001).

Table 1: *Results of ANOVAs for the effects of temperature and oviposition day on fecundity, egg size, number of unchorionated and chorionated oocytes, percent of chorionated oocytes and oocyte size in Bicyclus anynana. Significant P-values are given in bold.*

Trait	Source	Df	MS	F	P
Fecundity [n]	Temperature	1	32242.0	103.9	**< 0.0001**
	Day	5	5435.0	17.5	**< 0.0001**
	Temp. x day	5	1450.6	4.7	**0.0004**
	Error	444	310.2		
Egg size [mm^2]	Temperature	1	0.115	45.5	**< 0.0001**
	Day	5	0.002	0.7	0.6610
	Temp. x day	5	0.005	1.9	0.0967
	Error	415	0.003		
Unchorionated oocytes [n]	Temperature	1	0.034	0.1	0.7740
	Day	4	3.622	8.9	**< 0.0001**
	Temp. x day	4	0.893	2.2	0.0704
	Error	182	0.405		
Chorionated oocytes [n]	Temperature	1	0.010	< 0.1	0.8862
	Day	4	2.598	5.2	**0.0006**
	Temp. x day	4	0.547	1.1	0.3621
	Error	184	0.501		
Chorionated oocytes [%]	Temperature	1	6.8	< 0.1	0.8881
	Day	4	1223.9	3.6	**0.0078**
	Temp. x day	4	153.4	0.5	0.7736
	Error	184	342.2		
Oocyte size [mm^2]	Temperature	1	0.006	1.1	0.2897
	Day	4	0.012	2.5	**0.0472**
	Temp. x day	4	0.008	1.7	0.1497
	Error	184	0.005		

Experiment 2

In line with the above results, the number of chorionated and unchorionated oocytes and their percentages on oviposition day 12 was not affected by temperature (Tables 2, 3), though chorionated and unchorionated oocytes weakly tended to be more numerous at 27°C compared to 20°C. Mating, on the other hand, significantly

decreased the number of chorionated and unchorionated oocytes at both oviposition temperatures, and also decreased the percentage of chorionated oocytes in mated compared to virgin females.

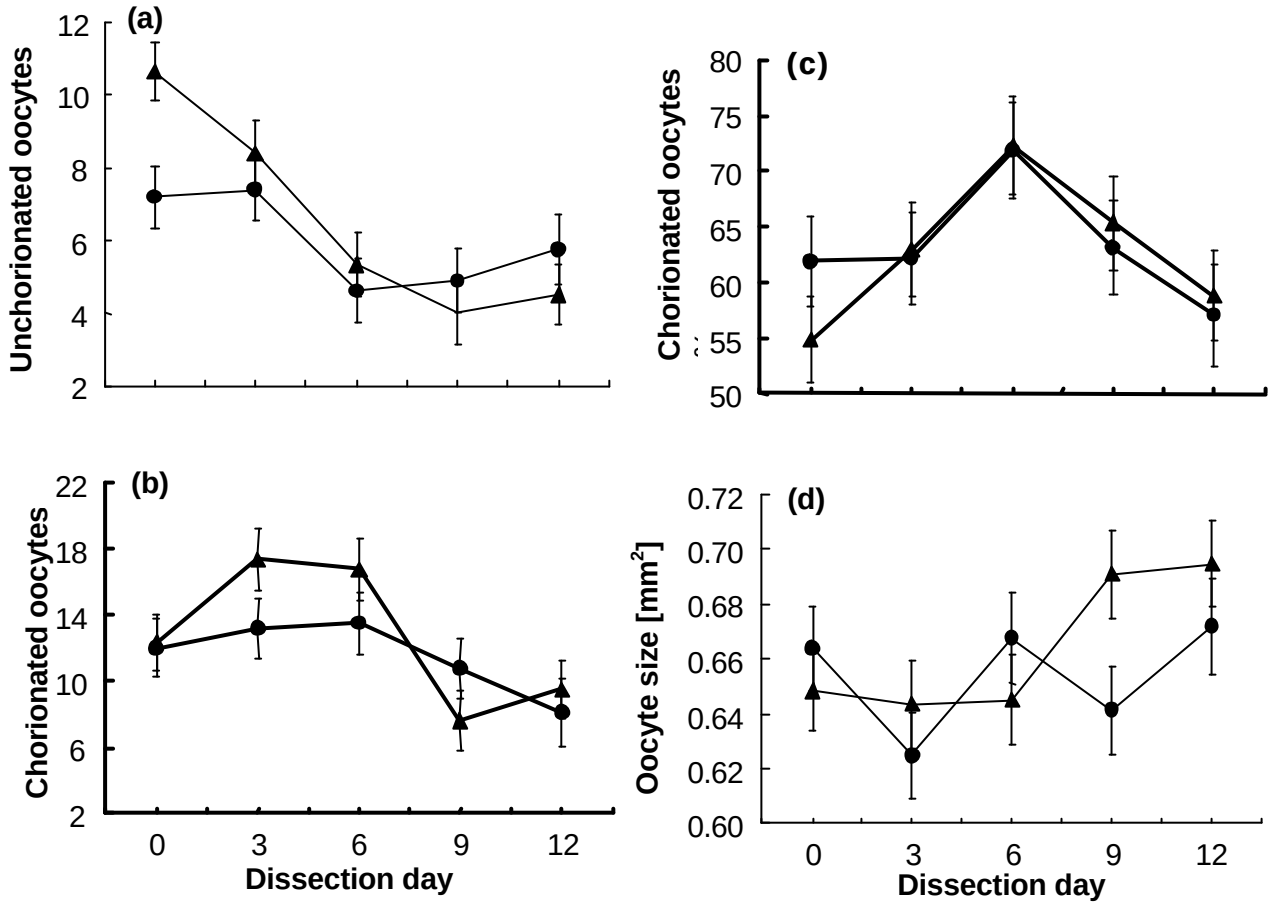

Figure 2. *Number of unchorionated oocytes (a), chorionated oocytes (b), percentage of chorionated oocytes (c), and oocyte size (d) over time in Bicyclus anynana females kept at 20°C or 27°C. Dissection day 2 equals day 6 of adult life. Given are means ± 1SEM. Triangles: 20°C, circles: 27°C.*

Oocyte size was affected by both, mating status and temperature, with virgin females having larger oocytes than mated once, and females ovipositing at 27°C smaller oocytes than those ovipositing at 20°C. The temperature effect was much more pronounced in mated compared to virgin females (significant interaction term; Tables 2, 3).

Table 2. *Number of unchorionated and chorionated oocytes, percentage of chorionated oocytes and oocyte size (means ± SEM) in mated and virgin Bicyclus anynana females at 20°C and 27°C on oviposition day 12.*

	Virgin		**Mated**	
	20°C (n = 43)	**27°C** (n = 55)	**20°C** (n = 23)	**27°C** (n = 45)
Unchorionated [n]	7.3 (0.6)	7.8 (0.6)	2.3 (0.7)	3.2 (0.5)
Chorionated [n]	18.5 (1.2)	20.0 (1.1)	4.7 (1.3)	5.2 (1.0)
Chorionated [%]	72.0 (2.4)	70.4 (3.0)	60.01 (4.4)	51.5 (3.1)
Oocyte size [mm^2]	0.694 (0.010)	0.672 (0.009)	0.687 (0.014)	0.615 (0.010)

Table 3: *Results of ANOVAs for the effects of mating status and temperature on the number of unchorionated and chorionated oocytes, percentage of chorionated oocytes and oocyte size in Bicyclus anynana. Significant P-values are given in bold.*

Trait	**Source**	**Df**	**MS**	**F**	**P**
Unchorionated oocytes [n]	Mating	1	72.2	92.1	**< 0.0001**
	Temperature	1	2.8	3.6	0.061
	Mating x Temp.	1	1.1	1.5	0.2297
	Error	188	0.8		
Chorionated oocytes [n]	Mating	1	306.8	201.7	**< 0.0001**
	Temperature	1	1.4	0.9	0.3448
	Mating x Temp.	1	0.2	0.1	0.7256
	Error	188	1.5		
Chorionated	Mating	1	9483.9	18.8	**< 0.0001**
oocytes [%]	Temperature	1	1063.4	2.1	0.1487
	Mating x Temp.	1	494.0	1.0	0.3242
	Error	171	505.4		
Oocyte size [mm^2]	Mating	1	0.038	8.8	**0.0034**
	Temperature	1	0.081	18.8	**< 0.0001**
	Mating x Temp.	1	0.024	5.7	**0.0184**
	Error	162	0.004		

Discussion

In line with results from previous studies, female *B. anynana* exhibited temperature-mediated plasticity in egg size and number, producing higher numbers of smaller eggs at the higher temperature, but fewer and larger eggs at the lower temperature (Fischer *et al.* 2003a,c). The initial delay in the response of egg size to temperature was expected and suggests that a temperature change causes a gradual response, stretched out over several days (Fischer *et al*. 2003a,b,c, 2004). Similar plastic responses in egg size were also found in some other arthropods (e.g. Azevedo *et al*. 1996; Crill *et al*. 1996; Ernsting & Isaaks 1997; Blanckenhorn 2000). Such differences in egg size cannot be attributed to differences in body size or physiology during development (Moore & Folt 1993), but are directly caused by differences in the adult thermal environment. Consequently, this plastic response in egg size was found to be reversible (Fischer *et al*. 2003a,b). This study was aimed at disentangling the developmental mechanisms underlying temperature-mediated variation in egg size and number, by investigating ovarian dynamics in addition to reproductive output across temperatures.

Given that, at least in *B. anynana*, there is evidence for such plasticity representing adaptive phenotypic plasticity (Fischer *et al*. 2003a,b, 2004; Steigenga *et al*. 2005), unravelling those underlying mechanisms is an important task to gain a more integrated understanding of temperature effects on phenotypic expression (French *et al*. 1998; Azevedo *et al*. 2002; Calboli *et al*. 2003).

Regarding reproductive output, lifetime fecundity was roughly twice as high at 27 compared to 20°C (cf. Fischer *et al*. 2003a). This, however, does not reflect a trade-off between egg size and number, because variation in egg size is relatively small compared to that in egg numbers. Accordingly, total reproductive investment increased at the higher temperature (cf. Avelar 1993; Ernsting & Isaaks 1997, 2000; Fischer *et al*. 2003a). These findings challenge the idea of reduced costs of somatic maintenance at lower temperatures, enabling the allocation of more resource to reproduction and consequently larger egg sizes (Avalar 1993; Fox & Czesak 2000). The lack of differences in oocyte numbers across temperatures (see below) further suggests that reduced fecundity at lower temperatures is not caused by delayed oviposition (Wallin *et al*. 1992; Huey *et al*. 1995), as oocytes did not accumulate in

the ovaries or oviducts in females ovipositing at the lower temperature. This is particularly obvious in the comparison between mated and virgin females (Table 2).

Regarding ovarian dynamics, the numbers of unchorionated oocytes dropped from an initial peak to lower levels during oviposition, while numbers of chorionated oocytes were highest during oviposition days 0-6. The percentage of chorionated oocytes increased during early oviposition due to chorionisation, followed by a later decline (cf. Satyanarayana *et al*. 1991, 1992; Zeng *et al*. 1997; Delisle & Cusson 1999; Webb *et al*. 1999). Comparing patterns of ovarian dynamics and realized fecundity indicates that the number of oocytes present in the ovaries seems to be a reliable indicator for daily fecundity (cf. Ernsting *et al*. 1992). In contrast to these (expected) patterns over time, oocyte numbers did not differ between temperatures in both experiments (cf. Ernsting *et al*., 1992), while the size of terminal oocytes clearly increased at the lower temperature (cf. additional data in *experiment 1* and *experiment 2*). The inconclusive results from *experiment 1* are likely to represent a methodological artifact, based on not exclusively measuring terminal oocytes (see Methods). Blanckenhorn & Henseler (2005) reported a decrease in ovariole volume at high temperatures in the dung fly *Scatophaga stercoraria.* But whether this was due to a change in the number and/or size of the oocytes remained unclear.

Mating (and host-plant availability) had pronounced effects on egg development, reducing the number of chorionated and unchorionated oocytes, and decreasing the percentage of chorionated oocytes in mated compared to virgin females. These findings suggest that mating is not necessary to induce egg development, but that mating functions as an important stimulus for egg deposition, without which virgin females retain their eggs as long as possible. Based on the fact that *B. anynana* is effectively unable to completely inhibit oviposition (Fischer in review), the ongoing maturation of additional eggs, combined with a reluctance to oviposit, results in an accumulation of oocytes in the ovaries (cf. Herman & Barker 1977). Interestingly, temperature effects on oocyte size were much more pronounced in mated than in virgin females, probably reflecting low levels of egg deposition in virgin females resulting in generally larger eggs (cf. Bauerfeind & Fischer 2005).

Conclusions: Developmental mechanisms and egg size plasticity

Our results demonstrate clear effects of oviposition temperature on egg (plus oocyte) size and fecundity in *B. anynana*, not affecting ovarian dynamics in terms of oocyte numbers or the ratio of chorionated versus unchorionated oocytes. What does that mean regarding underlying developmental mechanisms? Firstly, based on a comparison across temperatures and between mated and virgin females, we can clearly rule out that the production of larger eggs at lower temperatures result from delayed oviposition (e.g. Wallin *et al*. 1992; Huey *et al*. 1995). There was not the slightest evidence for an accumulation of oocytes in females ovipositing at lower temperatures (see above).

Given equal numbers of oocytes in the ovaries across temperatures at any given time but much reduced egg laying rates at the lower temperature, our data suggest reduced oocyte production (i.e. differentiation) rates at lower temperatures, as has been previously proposed (Van der Have & De Jong 1996; Ernsting & Isaaks 1997 2000). As temperature generally slows down physiological processes in insects, resulting in reduced growth rates and extended development times, such reduced differentiation rates are likely to be accompanied by prolonged egg maturation times. Though both processes will jointly cause lower egg-laying rates (as observed), they do not *per se* affect egg size. If, however, oocyte growth (vitellogenesis) would be (even only marginally) less sensitive to temperature (Van der Have & De Jong 1996; Blanckenhorn & Henseler 2005) as compared to oocyte production rate and egg maturation time, both, reduced egg numbers and larger egg size would result at lower temperatures.

Consequently, our results support the notion of a differential temperature sensitivity of oocyte production (i.e. differentiation) versus vitellogenesis (i.e. growth), as postulated earlier by Van der Have & De Jong (1996) and Ernsting & Isaaks (1997 2000). This hypothesis is based on a biophysical model. While growth depends in the first place on the rate of protein synthesis, differentiation depends primarily on the rate of DNA replication. Based on differences in molecule sizes and concomitant differences in temperature-dependent diffusion rates, the model predicts intrinsic differences in the temperature coefficients for growth (gain in biomass, cell size) and differentiation (DNA replication, cell numbers; Van der Have & De Jong 1996). The

biophysical model, supported here by empirical evidence, identifies temperature constraints on growth and differentiation. This, however, does not rule out that such physiological processes may have been exploited and thus further shaped by natural selection, resulting in overall adaptive egg sizes (Van der Have & De Jong 1996, Van Voorhies 1996, Ernsting & Isaacs 1997; Fischer *et al*. 2003, Blanckenhorn & Henseler 2005; Walters & Hassall 2006).

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7. Publication list

Chapters 5 and 6 have been published in international peer-reviewed journals as follows:

Subchapter 5.1

Steigenga, M. J., Zwaan, B. J., Bakefield, P. M. and Fischer, K. 2005. The evolutionary genetics of egg size plasticity in a butterfly. *Journal of Evolutionary Biology*, **18**, 281-289.

Subchapter 5.2

Steigenga, M.J. and Fischer, K. 2007. Within- and between-generation effects of temperature on life-history traits in a butterfly. *Journal of Thermal Biology*, **32**, 396-405.

Subchapter 6.1

Steigenga, M.J., Hoffmann, K.H. and Fischer, K. 2006. Effects of the juvenile hormone mimic pyriproxyfen on female reproduction and longevity in the butterfly Bicyclus anynana. *Entomological Science*, **9**, 269-279.

Subchapter 6.2

Steigenga, M.J. and Fischer, K. 2007. Ovarian dynamics, egg size, and egg number in relation to temperature and mating status in a butterfly. *Entomologia Experimentalis et Applicata*, **125**, 195-203.

8. Contributions

All experiments and literature survey were conducted by myself – if not stated otherwise (see below). Chapters 1 to 4 providing a general introduction, synopsis, and the summary of this thesis were written by myself as were all published articles of this thesis (chapters 5 and 6).

All subchapters of chapter 5 and 6 have been published in international peer-reviewed journals with the following co-authors:

Prof. Dr. Klaus Fischer is the supervisor on my thesis and co-author of all publications. He supported and supervised all stages of the projects, discussion of experimental design, analysis and discussion of the results and first drafts of the chapters.

Chapter 5, subchapter 5.1 Co-Authors **Dr. Bas J. Zwaan and Prof. Dr. Paul M. Brakefield** generously provided me with facilities at Leiden University, The Netherlands and contributed to the analysis, discussion and first drafts of the chapter.

Chapter 6, subchapter 6.1 Co-Author **Prof. Dr. Klaus H. Hoffmann** contributed laboratory facilities and assisted with the design of the experiments and the first drafts of the chapter.

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Chapter 6. subchapter 6.2. Experiments were conducted and analysed by myself with the help of Volker Bornitzky who reared the maize and Stephanie Bauerfeind, Thorin Geister, Susann Janovitz, Isabell Karl and Claudia Pflicke who assisted with caterpillar- and butterfly rearing.

Marc Steigenga

Bayreuth, July 2008

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10. Curriculum vitae

Marc Johan Steigenga

Personal data	Date of birth	01/14/1977	
	Place of birth	Rotterdam / The Netherlands	
	Nationality:	Dutch	
Education	**PhD student**		2003 - present
	University of Bayreuth, Germany		
	PhD Thesis:		
	Adaptation or physiological constraint:		
	Temperature-mediated plasticity in egg size		
	Supervisor: Dr. Klaus Fischer		
	Student in Biology		1996 - 2002
	Leiden University, The Netherlands		
	Diploma Thesis:		
	A hormonal approach to decoupling of development		
	Time and eyespot size in Bicyclus anynana.		
	Supervisor: Prof. Dr. P.M. Brakefield		
	Highschool		1990 - 1996
	Libanon Lyceum,		
	Rotterdam, The Netherlands Netherlands		

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden

Marc Steigenga

Bayreuth, Juli 2008